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SENSORY MORPHOLOGY AND CHEMICAL ECOLOGY OF THE STABLE FLY,
STOMOXYS CALCITRANS: HOST-SEEKING AND OVIPOSITIONAL SELECTION

By

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Under the Supervision of Professors

Frederick P. Baxendale and Junwei J. Zhu

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SENSORY MORPHOLOGY AND CHEMICAL ECOLOGY OF THE STABLE FLY,
STOMOXYS CALCITRANS: HOST-SEEKING AND OVIPOSITIONAL SELECTION

Khanobporn Tangtrakulwanich, Ph.D.

University of Nebraska, 2012

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Stable flies cause stress and discomfort to cattle, and other mammals, including humans and pets. Economic losses from stable flies to the U.S. cattle industry from loss of milk production and cattle weight gain exceed \$2 billion annually.

Traditional stable fly management employing sanitation and insecticides is costly and often fails to provide acceptable levels of control. A novel method for managing stable flies involves the use of attractants and repellents. This approach could be used to enhance the current level of stable fly control and ultimately be incorporated into a sustainable stable fly management program.

Gravid females' stable flies use chemical cues from host animals and the environment to locate suitable hosts, mates, and oviposition sites. Understanding stable fly olfaction and the electrophysiological responses to host seeking and ovipositional stimuli is essential for exploring stable flies' behaviors stimulated by odor cues. This study characterized the number, location and types of stable fly sensilla on antennae. Accompanying electroantennogram studies assessed antennal responses to host-associated volatile compounds. The attractiveness / repellency of selected naturally occurring compounds to stable fly feeding and oviposition were investigated under

laboratory and field conditions. Four majors' types of sensilla were documented: basiconic, clavate, coeloconic, and trichoid with three subtypes. Results of electroantennogram studies revealed significant antennal responses to host-associated compound; for example, 1-octen3-ol, phenol, *p-cresol*, indole, and dimethyltrisulfide. Catnip (*Nepeta cataria* L.) oil and its constituent nepetalactones compounds provided significant repellency of stable flies. Combinations of host derived volatile compounds (phenol and *m*- or *p*-cresol) were more attractive to stable flies than a single compound alone.

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Chapter I Introduction and Literature Review

Chapter I Introduction and Literature Review

INTRODUCTION

Stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae) are important pests of livestock, but especially cattle, poultry, and hogs. Large numbers of flies irritate the animals causing stress and restlessness leading to reductions in weight gain and feed efficiency which results in decreased production of meat, milk, and eggs (Cambell et al. 1987). Stable flies are also considered serious pests of confined cattle in feedlots and dairies (Bruce and Decker 1958, Campbell et al. 1977, Weiman et al. 1992), and can be serious pests of grazing cattle in pastures and rangeland in the Northern Great Plains (Campbell et al. 2001). Stable flies are cosmopolitan, but are most abundant in temperate zones (Skidmore 1985) and are often associated with horses and cattle (Morgan et al. 1983).

Stable flies can also be important temporary pests of humans (Cilek 2008). They have been a significant problem in coastal areas on New Jersey, along the shores of Lake Superior and several Tennessee Valley Authority lakes, and near the beaches of northwest Florida (Cilek 2008). In addition, stable flies are also pests of humans in the ever-expanding rural-urban interface (Hall and Smith 1986, Thomas 1993).

Both sexes are blood feeders that prefer feeding on the animal's legs (Feddes et al. 1985). Their feeding behavior makes them potential vectors of animal pathogens (James and Harwood 1969). Mechanical transmission of trypanosomes by stable flies has been documented many times in laboratory rodent models (Wells 1972). Sumba et al. (1998) showed that contamination of mouthparts is sufficient for mechanical transmission

of trypanosomes by *Stomoxys* species, but transmission by regurgitation may also play a role (Macedo 2004).

The most effective method of controlling stable fly involves scattering their breeding habitat. This permits breeding materials to dry and denies the flies a moist oviposition site. Sanitation involving the removal of organic waste is also a useful strategy. However, these approaches are time consuming and costly (Skoda 1992). Insecticide applications have been the main strategies for preventing or reducing stable fly infestations in both rural and urban areas (Zumpt 1973, Campbell and McNeal 1979). Insecticide applications can provide short term protection to the cattle, but control is generally limited because flies do not remain on the animals after feeding.

Developing effective pest management strategies requires a comprehensive understanding of the conditions under which immature stable flies develop (Patterson 1981). Further, understanding how gravid females select suitable habitats to lay their eggs to maximize larval survival is important. Reudler Talsma et al. (2008) point out that the oviposition choices of an insect are based on a complex set of stimuli and associated responses. For blood sucking insects, selection of a suitable oviposition site is primarily based on olfactory and visual cues (Seenivasagan and Vijayaraghavan 2010). Gravid haematophagous females usually detect suitable oviposition substrates using odor receptors on the antennae and contact chemoreceptors on the tarsi, mouthparts and antennae (Seenivasagan and Vijayaraghavan 2010).

Despite the economic importance of stable flies, relatively little is known about how they response to host and ovipositional stimuli. Gathering information on the morphology and physiology of stable fly olfactory organs is an important first step in

identifying potential attractants and repellents which could ultimately be used in a stable fly integrated pest management program. Morphological studies would provide information on the location, number, and pattern of olfactory sensilla. In addition, by comparing sensilla morphology it is possible to classify types of sensilla housing olfactory cells sensitive to attractive or repellent odors. For example, most receptor cells in trichoid sensilla of the male giant sphinx moth, *Manduca sexta*, are specialized for detecting sex pheromones, leading to mate-seeking behavior (Hildebrand 1995). The presence or absence of similar structures should be studied in stable flies.

This research focused on the morphology and physiology of the stable fly olfactory system using electron microscopy and electrophysiological techniques. In addition, attractive or repellent odor compounds were chemically analyzed and their key components were described. The specific objectives of this research were to:

- 1) Identify the types and distribution of chemosensory hairs on stable fly antennae.
- 2) Document differences in the morphology of sensory hairs on male and female antennae.
- 3) Conduct electroantennogram analyses to document electrophysiological responses of stable flies to selected host animal and their environmental-related odorants.
- 4) Investigate the repellency/deterency of selected naturally occurring compounds to stable fly feeding and ovipositional behaviors under laboratory and field conditions.

LITERATURE REVIEW

Stable flies are serious economic pests of livestock including cattle in North America (Campbell and Hermanussen 1971, Christensen 1982). Because stable flies feed on blood from practically any warm blooded animal including horses, pets, humans and other livestock, they are considered pests of livestock and people. Infestations of 50 flies per cow on beef cattle can reduce weight gain by 25 percent and, in dairy cattle, decrease milk production by 40 to 60 percent (Tomberlin 2010).

The stable fly's official name is *Stomoxys calcitrans* (L.) (Stoetzel 1989), but it is also referred to as the “dog fly” (King and Lennert 1936), “biting house fly”, “wild fly”, “straw fly”, “stock fly” (Bishop 1913, 1920). Zumpt (1973) and Skidmore (1985) characterized the classification and nomenclature of stable fly as follows:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Diptera

Suborder: Cyclorrhapha

Division: Schizophora

Section: Calyptrate

Family: Muscidae

Subfamily: Stomoxyinae

Tribe: Stomoxini

Genus: *Stomoxys* Geoffroy 1762

Species: *calcitrans* Linnaeus 1758

Stable Fly Biology

Distribution

Stable flies have attained a worldwide distribution rivaling that of the house fly (*Musca domestica*). The distribution of this species is more common in temperate regions. It occurs in the tropics, but generally in lesser numbers than in cooler climates. This could be due to the presence of natural enemies including parasites and predators (Muir 1914). As 28 of 31 species are endemic to the Indo-Ethiopian region, it remains uncertain whether it was the temperate part of the Palearctic region that gave rise to this group as suggested by Brues (1913). Zumpt (1973) speculated that stable flies probably originated in tropical parts of the old world.

Morphology

The stable fly closely resembles the common housefly (*Musca domestica*). Unlike the house fly, however, *S. calcitrans* has a broader abdomen and piercing mouth part instead of sponging mouth part. Adult stable flies average 8 mm in length, have a gray body, and can be identified by four characteristic longitudinal strips across the thorax as well as several dark spots on the dorsal side of the abdomen. On the vertex and frons there are three ocelli and two large compound eyes. Stable flies display sexual dimorphism. There is more distance between the compound eyes in females. The proboscis of the stable fly is black, long, and thin and protrudes from the front of the head. The remaining mouth parts are modified with the labellum having rows of teeth in order to pierce the skin of its host. The palps are one third the length of the proboscis. Males and females are both blood feeders. Stable fly eggs are white, elliptical, about 1 mm long by 0.3 mm wide, and are laid in small clutches (25-50). Larvae range from 5 to

12 mm in length and are yellowish-white and cylindrical shape. Pupae have a reddish-to-dark brown exterior and are 4 to 7 mm long. The posterior spiracles on the puparia are black with three S-shaped yellow slits, and are lightly sclerotized (Bishop 1913).

Life cycle

Stable flies have a life cycle similar to house flies, but develop at a slightly slower rate. They are holometabolous. Females can lay up to 800 eggs over her life-span. The eggs take from one to four days to develop depending on the temperature, humidity, and how long the egg was retained by the female. The larval stage lasts from 11 to 30 (and sometimes more) days, based on habitat suitability and availability of food. Pupation occurs after 6 to 20 days. As with larval development, length of the pupal stage is dependent on food abundance and quality during larval growth (Bishop 1913). The entire life cycle (egg to adult) requires 13-18 days at temperature of 24°C to 30°C. Adult flies have a life-span of about 20 days (Jeanbourquin 2005). Mating may occur as early as 2 days after emergence, with most flies (89%) having mated by day 5. Copulation lasts 4-6 min and first matings are only 60% successful. Males inseminate more than one female (six on the average). Females mate only once (Harris et al. 1996).

Feeding habit

Both male and female stable flies depend on blood meals. Repeated blood meals are needed for survival and continued egg production (Jeanbourquin 2005). Stable flies are known to suck liquids from decaying fruits and other plant parts (Zumpt 1973), and nectar feeding is apparently common in stable flies. Nectar meals may provide the flies with immediate source of energy for flight activity but without a blood meal sperm transfer is very low or non-existent. For females fed only sucrose solutions, eggs will

develop only to stage I (Jones et al. 1985, Jones et al. 1992). Bailey and Meifert (1973) observed that, in general, adult stable flies only approach a host to feed. The female is anautogenous, requiring several blood meals to complete ovarian development. The average stable fly blood meal (25.8 mg) is three times its average body weight (8.6 mg) (Parr 1962). Normally, stable flies cannot obtain a full blood meal on a single host because of the defensive behavior elicited by its painful bite. Thus, flies typically alight repeatedly on the same host or fly from one animal to another until feeding is completed (Harwood and James 1979).

There are three phases in the stable fly's search for a host. The first phase involves the appetitive-searching phase. This is followed by the activation and orientation phases when the stable fly encounters chemical stimuli (kairomones) indicating the presence of a host. Attraction occurs when the insect (having located a host) begins to feed (Lehane 1991). Unlike most other blood-feeding insect which are nocturnal, stable flies are diurnal (Lehane 1991). Holloway and Phelps (1991) reported that stable flies have a bimodal diurnal feeding pattern, locating the host through CO₂ and octenol. Temperature was determined to be the most important weather factor with the greatest biting activity observed at approximately 30°C. At 14°C the flies were no longer attracted to host animals (Zumpt 1973).

Adult flies feed throughout the day, but the greatest activity on cattle occurs between 10 a.m. and 4 p.m. (Hoffman 1968). Stable flies have also been observed feeding at dawn and in the late afternoon under field conditions, but will feed at any time during the day light hours (Mitzmain 1913). Castro (1967) reported that stable flies predominately feed on larger animals including cattle, horses, hogs, sheep, goats, and

humans. Stable flies also bite dogs, especially on their ears (Hogsette et al. 1987). Stable flies typically feed on the lower extremities such as below the knees and hocks of host animals, but will move to the sides and back if fly numbers are high on the lower extremities (Foil and Hogsette 1994).

Vision

Stable flies show peaks of spectral sensitivity at 350-365 nm, 450-550 nm, and 625-640 nm (Agee and Patterson 1983, Lehane 1991). Holloway and Phelps (1991) observed that cotton cloth targets dyed with phthalogen blue attracted three times more *Stomoxys* spp. than alsynite fiber glass panels. Because equal numbers of stable flies were captured at an odor source (acetone, octenol, and CO₂) in the presence or absence of a visual target (Torr 1989), it was concluded that stable flies probably rely less on visual cue for host location. Moreover, *S. calcitrans* displays differential responses to shapes. In decreasing order of preference, these include: horizontal rectangle, circle, square, and vertical rectangle. The tendency to alight on a target is greater if the object is large, dark, and not striped (Gibson and Torr 1999).

Economic importance

Stable flies are important economic pests of livestock, especially on cattle in North America (Campbell and Hernanussen 1971). The economic importance of stable flies results from two factors: disturbance by biting activity and transmission of animal pathogens (Jeanbourquin 2005). Biting stable flies cause stress to cattle resulting in economic losses through reduced weight gain (Weiman et al. 1992) and poorer feed conversion (Campbell et al. 1987). Harwood and James (1979) noted the important sources of livestock losses when cattle were subject to mass attacks by the flies included

various forms of physical injury (mostly loss of blood), lower milk production, reduced vitality, and loss of pasturing time. When cattle are under attack by stable flies, they may stop feeding and crowd in bunches which leads to heat stress and weight loss (Foil and Hogsette 1994). Stable flies can also influence the productivity of dairy cattle through decrease in milk production. According to Bruce and Decker (1958), there is an average 0.7% loss of milk production per stable flies per cow. Campbell et al. (1987) found that the economic threshold for stable flies attacking feeder heifers was less than two stable flies per front leg. Reduced feed efficiency accounted for 88% of the total loss. Campbell et al. (2001) also reported a 7% reduction in weight gain per stable fly on grazing yearling cattle. If steer prices averaged \$1.98 / kg, the loss would be valued at \$33.26 per animal or 2.33 cents per fly (Talley 2008).

Because of its feeding behavior, the stable fly acts as a mechanical vector and may transmit a wide variety of pathogens ranging from trypanosomes, helminths, protozoans, and bacteria to viruses (Zumpt 1973). Pathogens acquired from infected hosts can survive in blood meal residues and remain on the fly's mouth parts (Foil et al. 1987). These pathogens are readily transmitted to the next host when flies alight for feeding. Stable flies are also important in the transmission of trypanosomes including *Trypanosoma evansi*, which causes surra in horses, cattle, dogs, and camels, and *T. equinum*, which causes "Mal de Caderas" in equines, cattle, sheep, and goats (Lehane 1991). *Stomoxys calcitrans* is also involved in the transmission of the nematode *Habronema magus*, a stomach worm of equines (Lehane 1991). Morgan and Miller (1976) reported the successful mechanical transmission by *S. calcitrans* of hog cholera virus from infected to susceptible pigs.

Control of stable flies

The most practical and economical method for reducing stable fly populations is to eliminate or properly manage breeding sources. These activities include removing or dispersing breeding site substrates (e.g., rotting straw, manure, etc) and spraying cattle with repellents or applying contact insecticides to stable fly resting surfaces (Zumpt 1973). Releasing biological control agents such as parasitoids like *Spalangia nigroaenea* or *Muscidifurax zaraptor* which attack stable fly pupae can significantly reduce adult stable flies in cattle feedlots (Weinzierl and Jones 1998). The “push-pull” approach, which uses dispensers to modify natural host odors and make animals less attractive, can increase the number of flies caught in traps deployed in the vicinity (Birkett et al. 2004).

Olfaction in insects

The sense of smell is considered one of the oldest of our senses and is present in all phyla (Mustaparta 2002). Basically, olfaction is defined by the ability to perceive volatile substances. In insects, olfaction is a primary sense (Kaissling 1971) even though other modalities like vision, contact and proprio-reception play also crucial roles (Jeanbourquin 2005). The first example of the importance of olfaction in insects was the discovery of species specific sex pheromones in moths produced by females and that attract males for reproduction (Schneider 1992). Numerous studies have been conducted on perception of the external environment by insects and the role of odors in the location of food, habitat and oviposition sites. The perception of odor is accomplished by receptors (olfactory sensilla) mainly located on the antennae and palps (Jeanbourquin 2005).

Olfactory sensilla morphology

Insects detect odors using olfactory receptor cells that innervate sensilla located on the antennae and palps. Olfactory sensilla may have many different forms, but they all have openings in the sensillum walls (either pores or elongated slits) through which chemical molecules can reach the underlying dendrites. Two basic types of sensilla are present : single-walled and double-walled (Steinbrecht 1999). Single-walled sensilla include trichoid (long hairs) and basiconic (short hairs) form, whereas double-walled sensilla include coeloconic (pit-peg) and styloconic (subconical structure) forms. In single-walled sensilla, outer pores penetrate the cuticular wall and lead to several pore tubules that extend into the sensillum lymph-filled inner cavity. In double-walled sensilla, the sensillum hair wall consists of a palisade of hollow cuticular “fingers” which may be open or a partially fused (Kelling-Johannes 2001). The number of neurons in an olfactory sensillum varies among different types of sensilla, from two in the trichoid pheromone-specific sensilla of male moths to over twenty in the basiconic sensilla of grasshopper (Jeanbourquin 2005). Dendrites, which are the branched projection of a neuron act to conduct the electrochemical stimulation received from other neural cells to the cell body, or soma, of the neuron. The dendrite extends to the hair in the receptor lymph which has a high K^+ (potassium ion) concentration maintained by ion pumps (Kaissling and Thorson 1980). Because the antennal lobe is the place where it arborizes on an olfactory glomerulus (Hansson 1995, Mustaparta 1996), all the olfactory neurons which respond to the same compound will project their axons in the same glomerulus. This produces a topographic map of antennal receptor activation in the brain (Vosshall et al. 2000).

Odor reception system

The entry, exit, and residence time of odorant molecules in the receptor environment have been denoted as perireceptor events (Getchell et al. 1984). These perireceptor events correspond to the extracellular steps of the incoming odor. As most odorant compounds are lipophilic in nature they readily dissolve in the epicuticular lipids on the surface of the sensillum, but they will not dissolve in the insect's sensillum lymph (Jeanbourquin 2005). In order to reach the dendrite membrane, hydrophilic proteins (odor-binding proteins or OBPs) combine with the odor molecule (Jacquin-Joly and Merlin 2004). OBPs function in transporting odor molecules through the sensillum lymph towards the odorant receptors in the membrane of the dendrite (Vogt and Riddiford 1986, Van den Berg and Ziegelberger 1991).

The odorant receptors (OR) are G proteins (guanine nucleotide-binding proteins) which are families of proteins involved in transmitting chemical signals outside the cell, and causing changes inside the cell. They communicate signals from many hormones, neurotransmitters, and other signaling factors. These odorant receptors are coupled proteins with seven transmembrane regions (Breer 1994). When the complex OBP reaches the dendrite surface, the odor molecule interacts with a receptor site. It remains unclear if the complex binds to the receptor or if the odor detaches from the OBP to complex with the receptor (Hansson 1995, Jacquin-Joly and Merlin 2004). The binding initiates a cascade of events inside the dendrite leading to the nervous activity. Binding of the odorant-OBP complex to the OR activates the G-protein, which couples to a phospholipase C (PLC). The PLC cleaves a membrane phosphatidyl inositol biphosphate (PIP₂), releasing the intracellular second messenger's inositol triphosphate

(IP₃) and diacylglycerol (DAG) (Bruch 1996). Through G- protein and phospholipase activation, inositol triphosphate (IP₃) is released in the cell and induces an increase of Ca^{2+} levels. The calcium activates a different secondary messenger system involving a protein kinase (PKC), which phosphorylates the ion channel involved in the depolarization of the chemosensory cell by opening cation channels (Hansson 1995, Mustaparta 2002, Jacquin-Joly and Merlin 2004)/ Upon depolarization of the dendrite, the receptor potential spreads electronically towards the nerve impulse generator on the soma of the receptor cells, and action potentials are generated and are sent via the axon to the brain (Kelling 2001). Some odors produce a hyperpolarization of the membrane, acting through a different second messenger system with cAMP (Chapman 1998).

The odor molecule must be removed from the receptor sites on the dendrites to allow detection of subsequent stimuli. Otherwise a high temporal resolution of changes in odor concentration and odor quality would not be possible (Stengl et al. 1999).

For deactivation of the odor, the odorant-OBP complex is rapidly oxidized, possibly by the receptor (Ziegelberger 1995). This oxidized odorant - OBP complex is unable to stimulate further receptor molecules. Finally, the odor is degraded by enzymes (Vogt and Riddiford 1986, Rybczynski et al. 1990, Pophof 1998).

Odor specificity

The insect olfactory system responds to and distinguishes myriads of volatiles. Even “unknown” chemicals are readily detected when they are encountered for the first time. The olfactory system may possess thousands of odorant receptors, or alternatively, the specificity may be based on fewer OR types, each reacting with a wide range of odorants (Breer 1994).

The specificity of a neuron is assumed to correlate with the presence of the receptor in the dendrite membrane. Neurons responding to a single compound may contain only one type of receptor with specific binding properties (shape, conformation). Conversely, neurons responding to different types of chemicals may have several types of receptors sites (Jeanbourquin 2005). Neurons responding to compounds of similar structure may have only a single receptor site that will bind with odor molecules with a characteristic structure. The fitting of the molecule with the receptor may induce a distinct firing rate and thus elicit different behavior (Chapman 1998).

Electroantennogram (EAG)

The electroantennogram (EAG) is an instrument that measures antennal output to the brain for a given odor, and is often used in electrophysiological studies of the insect olfactory system. The EAG, invented in 1956 by German biologist Dietrich Schneider, recorded the combined electrical activity of the neurons in the antenna responding to a particular stimulus. This technique was used to demonstrate that olfactory receptors were present on insect antennae (Chapman 1998). EAG is widely used in screening of insect pheromones by examining the responses to fractions of a compound mixture separated using gas chromatography.

Gas chromatography (GC)

Gas chromatography is a chromatographic that can be used to separate volatile organic compounds (Douglas 2010). A gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, a detector, and a data recording system. The organic compounds are separated based on differences in their partitioning behavior between the mobile gas phase and the stationary phase in

the column. Gas chromatography columns are of two designs: packed or capillary.

Packed columns consist of a glass or stainless steel coil (typically 1-5 cm total length and 5 mm inner diameter) filled with the stationary phase, or a packing coated with the stationary phase. The stationary phase is the part of the chromatographic system which the mobile phase flows where distribution of the solutes between the phases occurs. The stationary phase may be a solid or a liquid that is immobilized or adsorbed on a solid. In general immobilization by reaction of a liquid with a solid is used in liquid chromatography and absorption of a liquid on a solid is used in gas chromatography but there are many exceptions to both of these generalizations. The stationary phase may consist of particles (porous or solid), the walls of a tube (e.g., capillary) or a fibrous material (e.g., paper). Capillary columns are a thin fused-silica (purified silicate glass) capillary (typically 10-100 m in length and 250 μ m inner diameter) that has the stationary phase coated on the inner surface (10-50 nm thickness). Capillary columns provide higher separation efficiency than packed columns but are more easily overloaded with too much sample.

After the components of a mixture are separated using gas chromatography, they must be detected as they exit the GC column. The thermal-conductivity (TCD) and flame-ionization (FID) detectors are the two most common detectors on commercial gas chromatographs. Both are sensitive to a wide range of components, and both work over a wide range of concentrations. While TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different at detector temperatures from that of the carrier gas), FIDs are primarily sensitive to hydrocarbons (even more than TCDs). However, FIDs cannot detect water.

Both detectors are also quite robust. Since TCD is non-destructive, it can be operated in-series before an FID (destructive) detector, thus providing complementary detection of the same analytes. Other detectors are sensitive only to specific types of substances, or work well only in narrower ranges of concentrations (Douglas 2010).

The mobile phases are generally inert gases including helium, argon, or nitrogen. The injection port consists of a rubber septum through which a syringe needle is inserted to inject the sample. The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixtures. Because the partitioning behavior depends on temperature, the separation column is usually contained in a thermostat-controlled oven. Separating components with a wide range of boiling points is accomplished by starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components. Most columns contain a liquid stationary phase on a solid support. Separation of low-molecular weight gases is accomplished with solid adsorbents.

Mass spectrometry (MS)

Mass spectrometry employs the formation of gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. The principle behind mass spectrometry (MS) involves generating ions from either inorganic or organic compounds by any suitable methods, separating the ions by their mass-to-charge ratio (m/z), and to detecting the ions qualitatively and quantitatively by their respective m/z and abundance.

Mass spectrometry is different from most other analytical techniques including spectroscopy or spectrometry which investigate the non-destructive interactions between

molecules and electromagnetic radiation. Mass spectrometry detects the effects of ionizing energy on sample molecules when chemical reactions in the gas phase are consumed during the formation of ionic and neutral species. This technique is very sensitive and requires only trace amounts of the sample material are used in the analysis. A mass spectrometer converts sample molecules into ions in the gas phase, separates them according to their mass to charge ratio (m/z) and sequentially records the individual ion current intensities at each mass, i.e., the mass spectrum. If these ion current intensities are drawn in histogram form taking the most intense ion current as 100%, the values of m/z versus percentage relative intensity (% RI) is called a line diagram.

Gas chromatography coupled with mass spectrometry (GC-MS)

Even though the GC instrument is effective in separating compounds into their various components, only the GC instrument cannot be used for reliable identification of specific results but produces uncertain qualitative results (Douglas 2010). We have to use GC instrument to separate compounds and use the MS instrument to produce the output by drawing an array of peak on a chart which we call the “mass spectrum”. Each peak represents a value for a fragment mass. A peak’s height increases with the number of fragments detected with one particular mass.

Gas chromatography coupled with mass spectrometry (GC-MS) is a technique that combines these methods to identify a new array of substances in a test sample. This technique consists of a gas chromatograph (GC) coupled to mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. This makes it perfect set up for the analysis of hundreds of relatively low molecular weight compounds found in environmental materials.

In order for a compound to be analyzed by GC/MS, it must be sufficiently volatile and thermally stable (University of Bristol 2011). In addition, functionalized compounds may require chemical modification (derivatization), prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. Samples are usually analyzed as organic solutions consequently materials of interest (e.g., soils, sediments, tissues etc.) need to be solvent extracted and the extract subjected to various 'wet chemical' techniques before GC/MS analysis is possible.

The sample solution is injected into the GC inlet where it is vaporized and swept on a chromatographic column by the carrier gas (e.g., helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source (Figure 1) where compounds eluting from the column are converted to ions.

There are two potential methods for ion production. The most frequently used methods are electron ionization (EI) and the occasionally used alternative is chemical ionization (CI). For EI a beam of electrons ionize the sample molecules resulting in the loss of one electron. A molecule with one electron missing is called the molecular ion and is represented by M^+ (a radical cation). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion, it usually fragments producing further smaller ions with characteristic relative abundances that provide a model for that molecular structure. This information may be then used to identify compounds of interest and help

elucidate the structure of unknown components of mixtures. CI begins with the ionization of methane (or another suitable gas), creating a radical which in turn will ionize the sample molecule to produce $[M+H]^+$ molecular ions. CI is a less energetic way of ionizing a molecule hence less fragmentation occurs with CI than with EI, hence CI yields less information about the detail structure of the molecule, but does yield the molecular ion of the targeted compound; sometimes the molecular ion cannot be detected using EI, therefore the two methods complement one another. Once ionized, a small positive ion is used to repel the ions out of the ionization chamber. The next component is a mass analyzer (filter), which separates the positively charged ions according to various mass related properties depending upon the analyzer used. Several types of analyzer exist: quadrupole (Figure 2), ion traps, magnetic sector, time-of-flight, radio frequency, cyclotron resonance and focusing to name a few. The most common are quadrupole and ion traps. After the ions are separated, they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of the data produced, converts the electrical impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer (University of Bristol 2011).

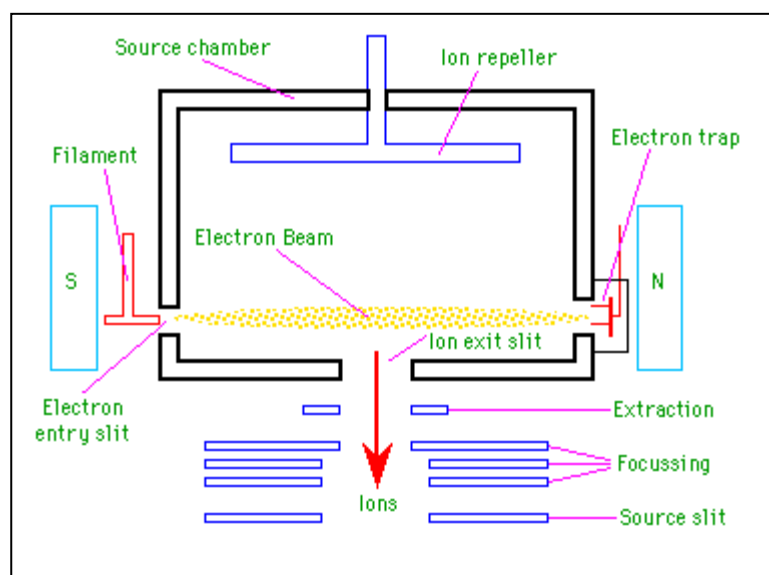


Figure 1 A schematic of an ion source

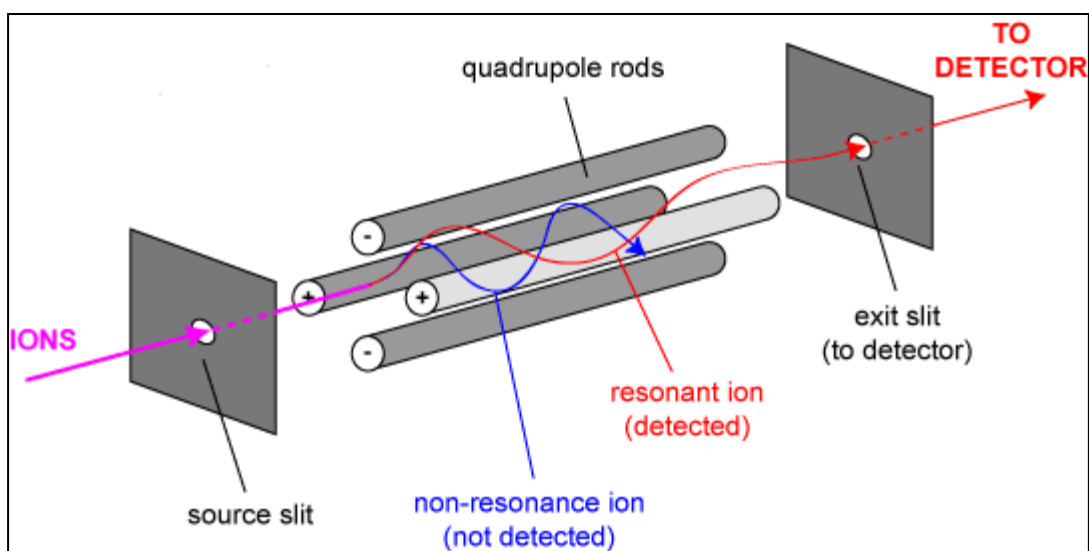


Figure 2 A schematic of a quadrupole analyzer

Diagrams by Dr. Paul Gates, School of Chemistry, University of Bristol

Volatile Collection

A conventional way of collecting volatile constituents from a solid involves adding a sample (e.g., manure, plant material, etc.) to a hermetic container such as sample vessel, heating it, and then collecting the constituents (gases) which evaporate from the sample and accumulate in the hermetic container. Another common way to collect volatile constituents is to heat the sample in a hermetic container which has a quality of being airtight and collect the evaporating constituents (gases) using a collecting agent provided in a collecting tube while the constituents (gases) are continuously circulated between the hermetic container and the collecting tube. This method of collecting volatiles consists of placing the sample in glass jar, blowing activated charcoal purified air (~200 ml/min) through the jar and collecting the volatiles on a Tenax trap connected to the outlet. These traps usually consist of a Pasteur pipet (5 cm long, 0.5 cm in diam.) which is packed with Tenax absorbent resin held in place by a glass wool plug. The volatiles are collected for a prescribed period of time (from a few minutes to few days, depend on the emission quantity), eluted with solvent, such as hexane, ether etc., and if necessary, the extracts have to be concentrated under a gentle nitrogen stream. Extracts are then analyzed using GC or GC-MS.

Solid phase microextraction (SPME)

Solid phase microextraction (SPME) is another technique for extracting volatile organic compounds. SPME was developed to address the need for a fast, solvent-free, and field compatible sample preparation method. It has been used for a wide range of applications including environmental, industrial hygiene, process monitoring, clinical, forensic, food and drug analysis (Pawliszyn 1997). In SPME, coated fibers are used to

isolate and concentrate analytes into a range of coating materials. After extraction, the fibers are transferred, with the help of a syringe-like handling device, to analytical instruments for separation and quantification of the target analytes. The analytical process consists of several steps including sampling, sampling preparation, separation, quantification and data analysis. When analyzing volatile compounds in water for example, the target analytes are first extracted into an organic solvent. The resulting solution is then introduced into an analytical instrument for separation, quantification, and identification. Each step affects the precision, accuracy, and speed of the analysis. Although multi-dimensional techniques for example, gas chromatography /mass spectrometry (GC/MS) have improved separation and quantification, however the preparation step is still time consuming and often uses a significant volume of organic sample. SPME was developed to simplify the preparation step by minimizing the amount of extraction solvent needed relative to the sample volume. The result that the amount of extraction is very small compared to the sample volume leads to inhibit exhaustive removal of analytes to the extracting phase. The equilibrium is rather reached between the sample matrix and the extracting phase instead. In order to make this technique applicative, the extracting phase is permanently attached to rods made from various materials. In most of the cases, the extracting phase is a polymeric organic phase that is cross-linked and permanently attached to the rod. In one configuration, the rods consist of an optical fiber made of fused silica, which is chemically inert (Figure 3). A polymer layer is used to protect the fiber against breakage. (Pawliszyn et al.19970). The polymers which have been used mostly are poly (imethylsiloxane) and polyacrylate. Poly acts as a liquid, which results in rapid extraction compared to polyacrylate, which is a solid. The

silica rods have a typical diameter of 100–200 microns and a film thickness ranging from 10–100 microns. When the coated fiber is placed in an aqueous matrix the analytes is transferred from the matrix into the coating. The extraction is presumably complete when the analytes have reached an equilibrium distribution between the matrix and fiber coating (Pawliszyn et al. 1997).

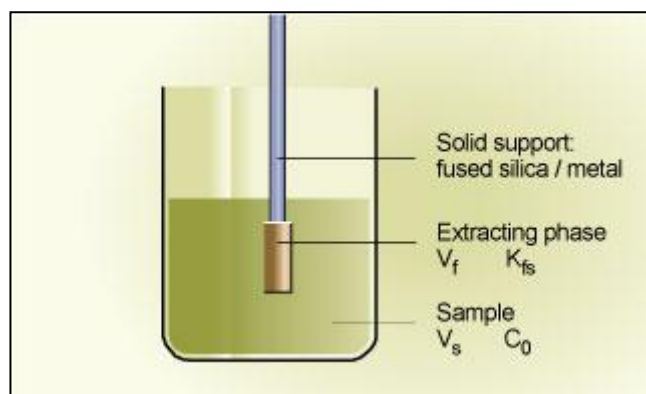


Figure 3 Micro-extraction with SPME

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**Chapter II Characterization of Olfactory Sensilla of *Stomoxys calcitrans* and
Electrophysiological Responses to Odorant Compounds Associated with their Host
and Oviposition Media**

ABSTRACT Stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), are economically important biting flies that have caused billions of dollars in losses in the livestock industry. Field monitoring studies have indicated that olfaction plays an important role in host location. To further our understanding of stable fly olfaction, we examined the antennal morphology of adults using scanning electron microscopy techniques. Four major types of sensillum were found and classified as: (a) basiconic sensilla; (b) trichoid sensilla with three subtypes; (c) clavate sensilla, and (d) coeloconic sensilla. No significant differences between male and female flies in abundances (total numbers) of these sensillum types were observed, except for medium-sized trichoid sensilla. The distinctive pore structures found on the surface of basiconic and clavate sensilla suggest their olfactory functions. No wall pores were found in trichoid and coeloconic sensilla, which suggests that these two types of sensillum may function as mechano-receptors. Details of the distributions of different sensillum types located on the funicle of the fly antenna were also recorded. Electroantennogram results indicated significant antennal responses to host-associated compounds. The importance of stable fly olfaction relative to host and host environment seeking is discussed. This research provides valuable new information that will enhance future developments in integrated stable fly management.

Key words. *Stomoxys calcitrans*, antennal morphology, electroantennogram (EAG), odorants, scanning electron microscopy (SEM), stable fly.

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INTRODUCTION

Stable flies, *Stomoxys calcitrans* L. are obligate, blood-feeding insects. They are considered significant economic pests of livestock and other warm blooded animals in many parts of the world (Zumpt 1973, Mullens et al. 1988, Masmehathip et al. 2006). Females lay eggs in decaying vegetable matter (including straw or hay) mixed with or without excrement from horses, cattle and sheep (Bishopp 1913, Pinkus 1913, Broce et al. 2005). Stable flies are known to use semiochemical for host location and selecting oviposition sites (Birkett et al. 2004, Jeanbourquin and Guerin 2007). To detect their hosts or oviposition sites, stable flies presumably use specific cues including visual and/or olfactory stimuli associated with the host and acceptable larval environments. Jeanbourquin and Guerin (2007) demonstrated that stable flies are able to locate either horse or cow dung by relying on odor cues, without contact with the substrate. Laboratory wind tunnel studies have shown that 1-octen-3-ol, 6-methyl-5-hepten-2-one and 3-octanol increases stable fly upwind flight, whereas naphthalene, propyl butanoate, linalool reduce upwind flight of the face fly, *Musca autumnalis*, D.G., horn fly, *Haematobia irritans*, L., screwworm fly, *Wohlfahrtia magnifica*, S., sheep headfly, *Hydrotaea irritans*, L. and the stable fly (Birkett et al. 2004). These findings indicate a critical role for olfaction used by flies to search for appropriate hosts and to avoid inadequate environments.

Sensory organs on the antennae of insects are known to be used in locating mates, hosts, habitats, and oviposition sites (Weseloh 1972, Vinson et al. 1986, Bin et al. 1989, Isidoro et al. 1996). With a few exceptions, studies of the antennal sensilla in Dipteran species have revealed an abundance of basiconic, coeloconic, and trichoid sensilla

(Castrejon-Gomez and Rojas 2009, Sutcliffe et al. 1990, Pfeil et al. 1994, Shanbhag et al. 1999, Fernandes et al. 2004, Sukontason et al. 2004, 2005).

In muscoid flies, most sensory organs used for the perception of chemical odorants are located on the funicle of antennae (Lewis 1970, White and Bay 1980, Bay and Pitts 1976). These sensory organs have been reported to respond to various stimuli such as warmth, humidity, skin odors, ammonia, and carbon dioxide (Krijgsman 1930, Hopkins 1964, Zdarek and Posisil 1965, Gatehouse 1969). Lewis (1971) using transmission electron microscopic (TEM) images described the internal structures of seven sensilla types and provided limited description of their external morphology. The present study characterized the morphology, abundance, and distribution of presumptive olfactory sensilla on the funicle of stable flies and their electrophysiological responses to selected host and oviposition site associated odorants.

MATERIAL AND METHODS

Scanning Electron Microscopy

Stable flies were obtained from the USDA-AMRU laboratory, Lincoln, Nebraska, USA, and were maintained at 23 ± 2 ° C with variable humidity (30-50% RH) and 12 L: 12 D photoperiod. Adults were fed citrated bovine blood (3.7g sodium citrate/litter) soaked in a feminine napkin (Stayfree[®], McNeil-PPC Inc., Skillman, New Jersey) placed on top of a screen cage.

The antennae of male and female stable flies (at least 10 from each sex) were observed using scanning electron microscopy. Stable fly heads were removed under a dissecting microscope (Olympus SZ-6, Olympus Inc. USA) using microsurgical scissors. The antennae were excised and fixed in 2.5% glutaraldehyde mixed with 0.1 M

Sorenson's phosphate buffer solution at a pH value of 7.4 at 4°C for 24 hours. Antennae were then rinsed twice with 0.1 M Sorenson's phosphate buffer and dehydrated with ethanol. The dehydration process was sequentially subjected to increasing ethanol concentrations of 30, 50, 70, 80, and 90%. Antennae were held in each concentration of ethanol for one hour during the dehydration process. Following dehydration, antennae were twice placed in absolute ethanol for a 12-hr period for further dehydration and then subjected to critical point drying. Dehydrated antennae were mounted vertically on aluminum stubs (allowing 360-degree imaging) and were coated with gold in a sputter coating apparatus. Samples were observed using a variable pressure scanning electron microscope and a field-emission scanning electron microscope (Hitachi 3000N and 4700, Japan) at the Microscopy Core Facility, Biological Technology Center, Beadle Center, University of Nebraska, Lincoln, Nebraska, USA.

Micrographs of the dorsal, ventral, outer side, inner side, and antennal tips of both males and female stable flies were taken at 500x magnification. The distribution and density of various sensilla types were determined using the grid technique described by Kelling (2001). The micrographs were divided into approximately 187 compartments and the number of sensilla was counted in each 1000 μm^2 compartment twice. Higher magnifications (up to 35,000x) were used to further investigate the fine structure of individual sensilla. The following procedures were used to obtain accurate sensilla maps: 1) comprehensive, large images (3500x) of the funicle were constructed by assembling a series of micro photographs (ranging in size from 30 to 60 compartments) into whole pictures of each antenna; 2) the antennal area was estimated from the number of compartments in the assembled images, and the mean number of sensilla per funicle

was calculated. The terminologies and nomenclatures used to describe antennal morphology and to classify sensilla types were adapted from Lewis (1971), Steinbrecht (1997) and Keil (1999).

Electroantennogram

Electroantennograms (EAG) were recorded by connecting an electrogel-filled (Spectro 360; Park Laboratory Parker, NJ) glass electrode to the excised head of a stable fly (as a ground contact). A recording electrode filled with the same electrode gel was connected to the tip of the funicle. Antennae were exposed to a charcoal filtered, humidified airstream of 0.5m/sec, and EAGs were recorded at room temperature ($25\pm1^{\circ}\text{C}$). The EAG system consisted of a high-impedance D.C. amplifier with automatic baseline drift compensation (SYNTECH Equipment and Research, Kirchzarten, Germany). An EAG program (SYNTECH EAG-Pro 4.6) was used to record and analyze the amplified EAG signals. Five hundred micrograms of each selected stable fly host associated odorant compounds was dissolved in 500 μl of redistilled HPLC-grade hexane. Ten microliters of the prepared solution were applied to filter-paper strips (0.5x 2.5 cm, Whatman No.1, Whatman International Ltd., England). The filter-paper strips (after air-drying) were inserted into Pasteur pipettes (15 cm in length). A 5 ml puff of odorant compound was blown through the pipettes and directed across the antennae to elicit an EAG response. Control puffs of air were applied after each puff of a test stimulus. The absolute EAG response of each stimulus was recorded as means from at least 6 replicated measurements. The sequence of exposure of each stimulus to each antenna was randomly defined.

Test odorant compounds

The test odorant compounds (indole, dimethyl trisulfide, 1-octenol-3-ol, phenol, *p*-cresol, 2-heptanone, acetic acid, butyric acid, isovaleric acid, and hexanoic acid) thought to be associated with stable fly host and oviposition sites were purchased from Sigma/Aldrich (St. Louis, Missouri, USA). Labeled purities for these odorants ranged from 98% to 99.5%.

RESULTS

Stable fly sensilla

Like most Muscidae, the antenna of the stable fly consists of three segments: a proximal scape, medial pedicle, and the funicle, the distal third antennal segment. Most sensilla were found on the funicle. Based on SEM observations, there were four major types of sensilla: one basiconic, three trichoid, one clavate, and one coeloconic (Fig.1). Trichoid sensilla types were grouped into three subtypes based on hair length (Fig. 1f).

Trichoid sensilla

Trichoid sensilla were the most abundant sensilla type on the funicle of both sexes. Based on the length, shape and surface morphology, three subtypes of trichoid sensilla were characterized as short (<9µm long), medium (12-15 µm long) and long (>20 µm long) (Fig.1f). The long trichoid sensilla had a smooth cuticular surface, but the short and medium ones had grooved surfaces (Fig.1g). The short-curved trichoid sensilla were the most common of the three subtypes. Most were on the inner and outer regions of the funicle with fewer (35%) distributed over the remainder of the funicle. Significantly more medium trichoid sensilla were found on males than females ($t= 9.3$, $P< 0.05$). No sexual dimorphisms were found in total numbers of sensilla for the other two subtypes (long

trichoids, $t=4.73$, $P<0.05$; short trichoids, $t=1.60$, $P<0.05$). No pore structures were identified on any of the trichoid sensilla types (Fig.1g).

Basiconic sensilla

The mean number of basiconic sensilla on the funicle of female and male stable flies was 1190 and 1149, respectively (Table 1). This type of sensilla had a basal diameter of 1.5-2.5 μm and its length was 4.7-5.0 μm with a blunt tip (Fig.1 c and 1h). The surface of these sensilla's wall was perforated by numerous pores of about 0.01 μm in diameter. Basiconic sensilla were mostly distributed on the ventral, outer and inner sides of the funicle (see Fig.2a).

Clavate sensilla

The length, shape, and size of clavate sensilla were similar to those of basiconic sensilla except they were distally enlarged (Fig.1d), and the average diameter of the tip of basiconic sensilla was 1.0 μm compared to 1.5 μm for clavate sensilla ($t=2.92$, $P<0.05$). There were significantly fewer clavate sensilla relative to those of basiconic and trichoid sensilla ($F=16.9$, $P<0.05$). There were a mean of 218 clavate sensilla on the funicle of the female antennae, while males had an average of 169. Like basiconic sensilla, clavate sensilla were also distributed mostly in the outer and inner regions, with relatively few located on other parts of the funicle. There were no significant differences in the total numbers of clavate sensilla between male and female stable flies ($t=6.0$, $P>0.05$).

Coeloconic sensilla

Coeloconic sensilla were the shortest (0.2-0.25 μm) and least numerous types of sensilla on the funicle. They arose from a wide base cone and had 9-12 cuticular fingers meeting at the distal tip. The shaft of a coeloconic sensillum was longitudinally grooved (Fig.1e).

They were most abundant on the outer region of the funicle and were sparse elsewhere (Table 1). Female and male stable flies had an average of 57 and 51 coeloconic sensilla, respectively.

Sensilla distribution

No differences in the distribution and abundance of sensilla types were detected between male and female stable flies, except the medium trichoid sensilla (Table 1). Trichoid and basiconic sensilla were the two most abundant types of sensilla, and were distributed on all surfaces of the funicle. Basiconic sensilla were embedded among trichoid sensilla. Over 70% of clavate sensilla were present on the inner and outer side of the funicle, with a few observed on the tip of the funicle. Half of coeloconic sensilla appeared on the outer side, and the rest located at the rest regions of the funicle. Figure 2 shows the distributions of each sensilla type on the stable fly funicle.

Electroantennogram results

Absolute EAG responses to ten selected odorant compounds associated with stable flies host and oviposition sites were shown in Figure 3. Significant EAG responses were elicited from stable fly antennae responding to all tested compounds, compared to the control ($t=2.96-22.6$, $P<0.05$). The average EAG response to the control from antennae of female and male stable flies was 0.15 ± 0.01 mV and 0.11 ± 0.01 mV, respectively. Highest EAG responses to 1-octen-3ol, indole, phenol and *p*-cresol were observed from both female and male antennae ($F=2.77$, $df=9, 42$, $P<0.05$ for females; $F=6.55$, $df=9, 46$, $P<0.001$ for males). No differences in EAG responses to the test compounds were found between sexes except that male antennae responded more strongly to butyric acid and isovaleric acid than did female antennae (for butyric acid, $t=$

2.77, $P < 0.05$; for isovaleric acid, $t = 2.79$, $P < 0.05$). Although a mean EAG at 500 μV elicited from female stable fly antennae responding to dimethyl trisulfide, compared to male response at 184 μV , no statistical difference was found ($t = 1.55$, $P = 0.09$)

DISCUSSION

In our study, three potential olfactory sensilla, basiconic, coeloconic and clavate were all found on the funicle of the antennae. However, the number and distribution patterns of these sensilla differed from those reported by Lewis (1971). While Lewis (1971) found an average of 3,300 basiconic sensilla on the funicle, our specimens had an average of 1,200 of this type of sensilla. In the Lewis (1971) study, most basiconic sensilla were located in the proximal lateral and ventral regions of the funicle whereas in our study basiconic and clavate sensilla were concentrated on the inner side region of the funicle. There were 5 times more basiconic sensilla than clavate sensilla found on the funicle, compared to the 30:1 ratio reported by Lewis (1971). Based on our SEM observations and Lewis' (1971) TEM images, there are approximately 700-1000 pores on each basiconic and clavate sensilla, suggesting an olfactory function. In most insects, olfactory sensilla are characterized by numerous pores in the sensilla cuticle that allows for the entry of odorant compounds (Steinbrecht 1996). The least common types of sensilla were coeloconic, which were mostly located on the outer side of the funicle. Coeloconic sensilla were the shortest in length and had a finger-like structure. Discrepancies between our study and those of Lewis (1971) may be due to differences in local fly populations (European versus American populations) and the use of scanning versus transmission electron microscopy.

Trichoid sensilla were the most abundant sensilla observed on the funicle of both stable fly sexes. The three subtypes of these thin, conical, and sharply pointed sensilla had either smooth or grooved surfaces and differed in length. Similar morphologies have been reported from other dipteran species (Ross and Anderson 1987, Rahal et al. 1996, Fernandes et al. 2004, Chen and Fadamiro 2008). Lewis (1971) estimated there were approximately 700 relatively thick-walled trichoid sensilla and a variety of thin-walled sensilla on the funicle. However, we counted over 16,000 trichoids on the funicle of both sexes. Since we did not find any pores on trichoid sensilla, we suggest a mechanoreceptor function for stable fly trichoid sensilla. Similar sensilla without surface pores are also found on the primary screwworm, *Cochliomyia hominivorax* C. (Fernandes et al. 2008), and the papaya fruit fly, *Toxotrypana curvicauda* G. (Arzuffie et al. 2008). Trichoid sensilla with a mechanical function have been reported for the parasitoid, *Microplitis croceipes* C. (Ochineng et al. 2000), human bot fly, *Dermatobia hominis* L. (Fernandes et al. 2002), and fire ants, *Solenopsis invicta* B. (Renthal et al. 2003). However, Lewis (1971), using transmission electron microscopy, observed about 500 pores per thick walled trichoid sensilla that have a general similarity to the long trichoids observed in this study. Trichoid sensilla with pores have also been reported in other fly species (Stocker 1994, Riesgo-Escovar et al. 1997, Shanbhag et al. 1999). Several studies have attributed an olfactory function for trichoid sensilla in *Drosophila* (Clyne et al. 1997, Riesgo-Escovar et al. 1997, Shanbhag et al. 1999), and Clyne et al. (1999) used electrophysiological studies to confirm pheromone sensitivity. It is surprising that significant differences in number of medium size trichoids are found between male and female antennae, which may indicate differences in antennal responses to different odors

as shown from EAG tests. However, our SEM study has shown that trichoids are not olfactory sensory. Further investigation is needed to reveal the function of medium size of trichoid sensilla.

Basiconic sensilla-were the second most abundant sensilla found on stable fly antennae. Basiconic sensilla have been reported as the most common sensilla type on male and female antennae of *Lucilia cuprina*, W., *Chrysomya megacephala*, F., *C. rufifacies*, *C. nigripes*, *Musca domestica*, L., *Synthesiomyia nudiseta*, W., and *Megaselia scalaris*, L. and *Trihopoda pennipes* F. (Giangiuliani et al. 1994, Sukontason et al. 2004). Three subtypes of basiconic sensilla were identified from *Pseudoperichaeta nigrolineata*, W., *Drosophila melanogaster*, M. and *C. hominivorax* (Rahal et al. 1996, Shanbhag et al. 1999, Fernandes et al. 2004), but we did not identify any subtypes of basiconic sensilla on stable fly antenna. In our study, clavate sensilla looked similar in morphology and in pore structure to those of basiconic sensilla, except with an enlarged tip. We found a pore density on the surface wall of both basiconic and clavate sensilla of ca. 35-40 pores per μm^2 . Our findings are in agreement with earlier studies on *S. calcitrans* (Lewis 1971) and *D. melanogaster* (Shanbhag et al. 1999). The presence of the pore structure on the surface wall of basiconic and clavate sensilla suggests their olfactory function, which will be further confirmed using single sensillum recording techniques. In *D. melanogaster* and *Phoracantha semipunctata*, F. similar sensilla were demonstrated to respond to specific odorant compounds (Siddiqi 1983, 1987, Lopes et al. 2002).

Coeloconic sensilla were the most distinctive (finger-like) and least abundant sensilla type (<0.3% of total sensilla) on stable fly funicle. They were mainly distributed along the outer side of the funicle. Coeloconic sensilla have 9-12 closely apposed

cuticular fingers. In *Dermatobia hominis*, L., coeloconic sensilla have pegs and are located in pits surrounded by microtrichia. However, in stable flies they arise directly from the surface of the funicle, which is similar to those found form in several *Drosophila* species (Fernandes et al. 2002, Riesgo-Escovar et al. 1997, Shanbhag et al. 1999). Similar types of coeloconic sensilla from many insect orders are not considered to have a chemosensory function (Yao et al. 2005, Steinbrech 1977). In our study, the absence of cuticular pores on coeloconic sensilla suggests they are unlikely to function as chemoreceptors. However, Schneider and Steinbrecht (1968) described coeloconic sensilla as olfactory receptors. Those sensilla were lack of pores on the surface wall, but had terminal tubule structures. Using single sensillum recording technique, Schneider and Steinbrecht (1968) recorded a response from this type of sensilla to CO₂, temperature, and humidity. Furthermore, pore channels, reported in grooves of similar sensilla on female antenna of *Aedes aegypti* were reported responding to lactic acid (Cribb and Jones 1995). The groves between the fingers at the distal half of coeloconic sensilla from the stable fly may also have the same chemosensory capacity. Further studies including TEM with negative staining and single sensillum recording are underway to demonstrate whether coeloconic sensilla function as chemosensory receptors, or not.

Stable flies use a wide variety of visual, olfactory, gustatory, and physical stimuli for host location (Zhu et al. 2008). Among these stimuli, volatile semiochemicals emitted from the host play a major role in mediating host location and oviposition site selection. Several cow urine, manure, and rumen-associated odorants have been identified that are attractive to stable flies (Logan and Birkett 2007, Jeanbourquin and Guerin 2007). Gravid stable fly females are capable of selecting an oviposition site based on microbe-derived

stimuli that indicate suitability of the substrate for larval development (Romero et al. 2006, Zhu et al. unpublished).

Three major types of stable fly attractants, derivatives of fatty acid and amino acids, and isoprenoids have been identified so far. 1-Octen-3-ol elicited the strongest EAG responses from both female and male stable fly antennae. This volatile, associated with rumen digesta compounds, has also been identified from cattle urine (Birkett et al. 2004, Jeanbourquin and Guerin 2007). Traps baited with 1-octen-3-ol have been reported to significantly increase stable fly catches (Holloway and Phelps 1991, Mihok et al. 1995). A second group of odorants associated with cattle manure and urine compounds, including phenol, *p*-cresol, dimethyl trisulfide and indole, also elicit strong EAG responses. These compounds, when produced by anaerobic bacteria isolated from aged horse manure, were attractive to gravid stable flies (Romero et al. 2006, Mohammed et al. 2003, Zhu et al. unpublished). Significant behavioral responses (activation and attraction) of stable flies were elicited by responding to lures containing 10 µg of dimethyl trisulfide, 1-octen-3-ol and *p*-cresol (alone/mixtures) in wind tunnel bioassays (Jeanbourquin and Guerin 2007). Although EAG responses were detected from stable fly antennae when tested with a range of straight and branched carboxylic acid compounds, their response levels were significantly lower than above cattle manure associated compounds. Among them, butyric acid and isovaleric acid showed the lowest EAG responses, which are similar to what found in Jeanbourquin and Guerin's study (2007). No differences were observed in EAG responses to most of tested compounds between the two sexes of stable fly antennae ($t = 0.42$, $P > 0.05$), except that significantly higher EAG responses were found from male antennae to butyric acid and female antennae to isovaleric acid

compared to their opposite sexes. However, why antennal responses of stable flies to these two particular compounds (25% of odorants identified from rumen digesta from Jeanbourquin and Guerin 2007) still remains mystery, further behavioral studies are under investigation for understanding how these cues are being used differently (host searching and oviposition site selection).

In conclusion, our study characterized the morphology and distribution of 6 different sensilla types on the funicle of stable flies. Three of them (basiconic, clavate, and coeloconic sensilla) may have potential olfactory chemoreceptor function. Trichoid sensilla are likely to be involved in mechanoreception only. Stable fly antennal sensilla are similar to those described from other muscoid flies. In general, there are no morphological differences on stable fly antennae for sensilla types. Significant EAG responses detected from stable fly antennae to some selected host associated volatile compounds indicate their use of olfactory cues for host and oviposition site searching. The characterization of morphological details and data on olfactory sensilla mapping lays the foundation for future trials using the single sensillum recording to advance our understanding of underlying the mechanisms of stable fly chemical ecology, sensory physiology and neuroethology. The knowledge gained from these studies will ultimately benefit the development of stable fly management strategies.

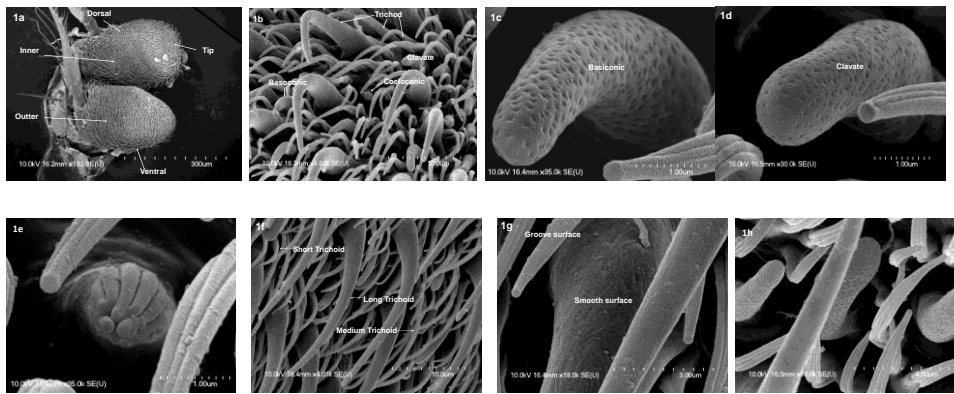


Figure 1 SEM micrographs of the stable fly antennae:(1a) Divided regions of sensilla type on antenna of *Stomoxys calcitrans*; (1b) Dorsal view of the funicle showing the distribution of all sensilla types; (1c-d) views showing shape differences between basiconic sensilla and clavate sensilla, with pore structures on the wall surface; (1e), High-resolution graph of sensilla coeloconica, (1f) 3 types of trichoid sensilla, (1g) close-up view showing smooth surface wall of trichiod sensilla, (1h) views showing the basal structures of basiconica and clavate sensilla.

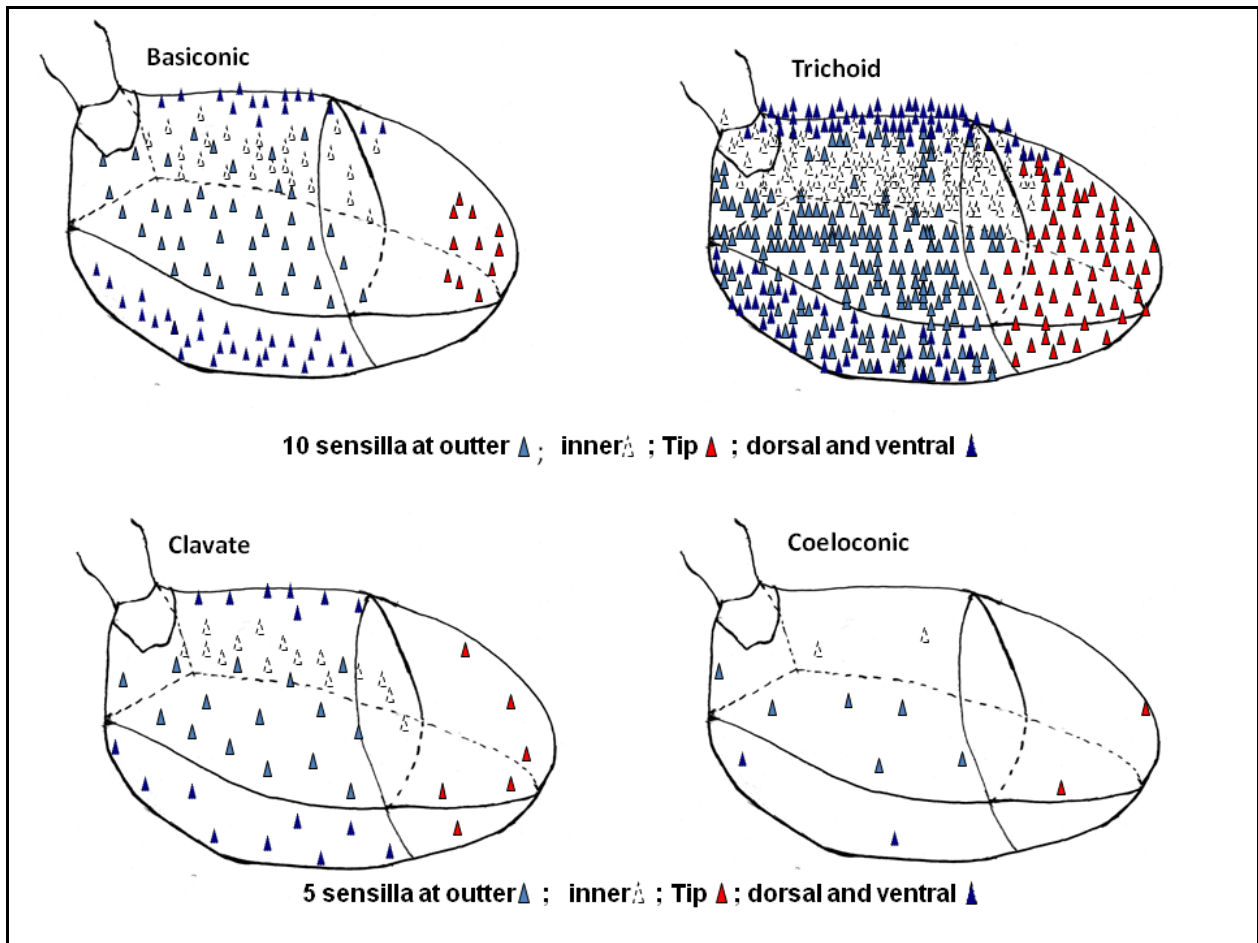


Figure 2 Constructed 3-D diagrams showing the distributions of the 4 principle types of sensilla on the funicle of *Stomoxys calcitrans*. A, basiconic sensilla; B, trichoid sensilla; C, clavate sensilla and D, coeloconic sensilla

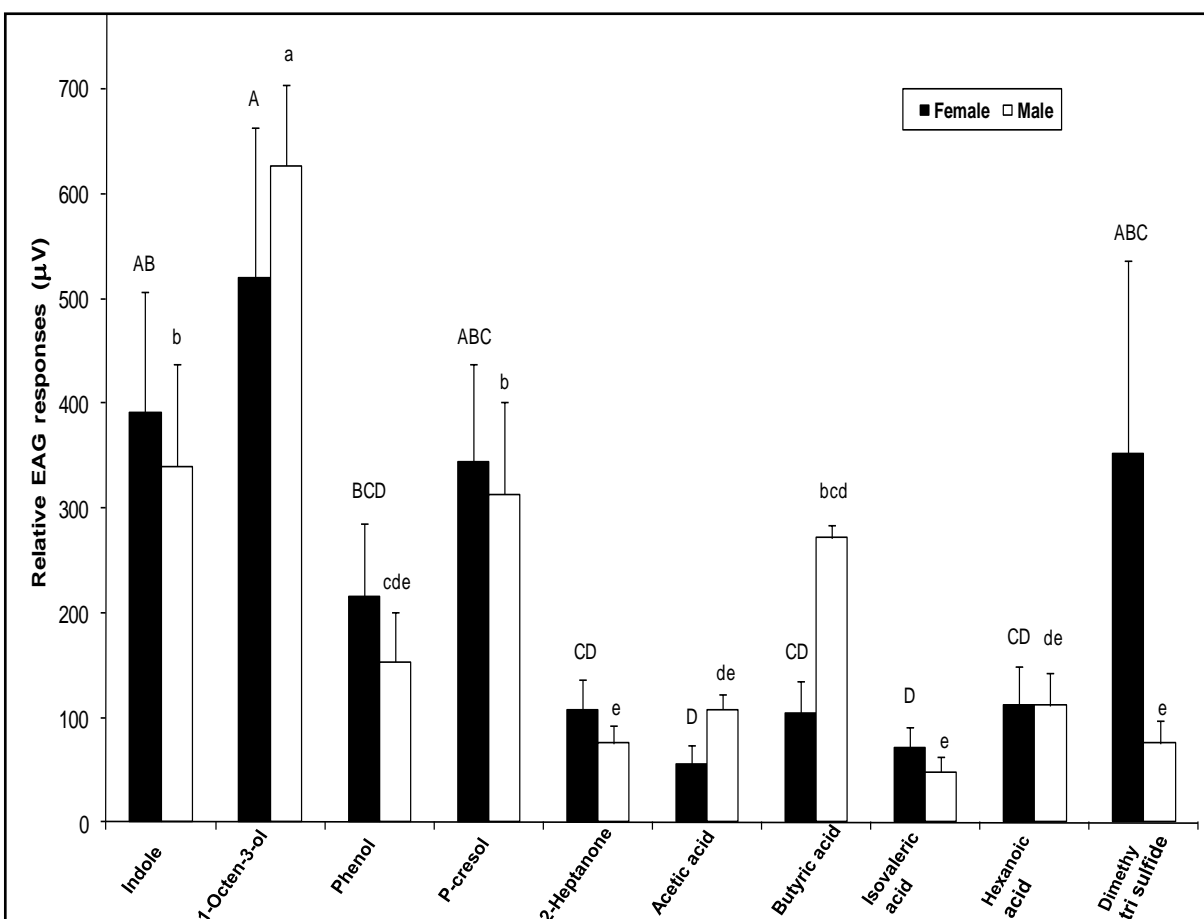


Figure 3 Relative EAG responses of male and female *Stomoxys calcitrans* to their host associated odorant compounds. Means with different letters above the bars are significantly different at $P < 0.05$ (SAS version 9.1, performed on the least-square means).

Table 1 Abundance and distribution of sensilla types on the funicle of the stable fly antenna (Mean \pm S.E.)

Area of funiculus		Basiconic	Clavate	Coeloconic	Trichoid (long)	Trichoid (medium)	Trichoid (short)
Tip	Male	100 \pm 34	6 \pm 1	5 \pm 1	611 \pm 2	302 \pm 32	587 \pm 86
	Female	159 \pm 36	26 \pm 6	9 \pm 4	542 \pm 204	379 \pm 79	1165 \pm 306
Dorsal	Male	120 \pm 40	10 \pm 6	1 \pm 1	371 \pm 78	549 \pm 76	949 \pm 269
	Female	158 \pm 18	31 \pm 27	1 \pm 1	626 \pm 142	852 \pm 161	1339 \pm 400
Inner side	Male	263 \pm 82	70 \pm 29	9 \pm 3	767 \pm 439	2157 \pm 1059	2542 \pm 535
	Female	238 \pm 39	41 \pm 5	10 \pm 4	907 \pm 590	1452 \pm 404	2316 \pm 1389
Outer side	Male	400 \pm 64	54 \pm 4	26 \pm 3	616 \pm 64	2566 \pm 273	3196 \pm 470
	Female	325 \pm 10	73 \pm 2	26 \pm 1	670 \pm 7	1028 \pm 4	3258 \pm 400
Ventral	Male	266 \pm 37	29 \pm 3	11 \pm 4	450 \pm 117	1651 \pm 267	1102 \pm 375
	Female	311 \pm 9	47 \pm 2	11 \pm 1	338 \pm 7	1002 \pm 4	996 \pm 4
Total	Male	1149 \pm 110	169 \pm 30	52 \pm 3	2815 \pm 462	7225 \pm 475*	8376 \pm 1025
	Female	1190 \pm 35	218 \pm 40	57 \pm 11	3082 \pm 382	4713 \pm 97	9072 \pm 1641

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**Chapter III Nepetalactones from essential oil of *Nepeta cataria* represent a stable
fly feeding and oviposition repellent**

ABSTRACT The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is one of the most serious pests to livestock. It feeds mainly on cattle and causes significant economic losses in the cattle industry. Standard stable fly control involving insecticides and sanitation is usually costly and often has limited effectiveness. As we continue to evaluate and develop safer fly control strategies, the present study reports on the effectiveness of catnip (*Nepeta cataria* L.) oil and its constituent compounds, nepetalactones, as stable fly repellents. The essential oil of catnip reduced the feeding of stable flies by >96% in an *in vitro* bioassay system, compared with other sesquiterpene-rich plant oils (e.g. amyris and sandalwood). Catnip oil demonstrated strong repellency against stable flies relative to other chemicals for repelling biting insects, including isolongifolenone, 2-methylpiperidinyl-3-cyclohexen-1-carboxamide and (1S,2_S)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide. The repellency against stable flies of the most commonly used mosquito repellent, DEET, was relatively low. In field trials, two formulations of catnip oil provided >95% protection and were effective for up to 6 h when tested on cattle. Catnip oil also acted as a strong oviposition repellent and reduced gravid stable fly oviposition by 98%.

Key words. *Nepeta cataria*, *Stomoxys calcitrans*, botanical-based repellent, oviposition repellent.

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L.), is one of the most problematic biting flies; it feeds mainly on bovids and equines in livestock barns, stables and pastures, and sometimes attacks pet animals and humans in cosmopolitan areas (Hafez and Gamal-Eddin 1959, Zumpt 1973). Bunching, the defensive behavior caused by stable fly attack on cattle, can result in reproductive failure and a reduction in meat and milk yields, with estimated economic losses of up to billions of dollars (Campbell et al. 1977, Stor 1979, Fraser and Broom 1990, D.B. Taylor, USDA-ARS, personal communication, 2011).

Although the stable fly is not considered an important disease vector, it is capable of transmitting a variety of pathogens, including helminths, protozoans, bacteria and viruses, some of which are primary agents of mortality in cattle (Zumpt, 1973, Buxton et al. 1985, D'Amico et al. 1996).

The control of the stable fly includes methods such as insecticide applications and cultural control. The long term use of toxic insecticides, however, is unsustainable and can lead to the development of insecticide resistance (Cilek and Greene 1994, Rozendaal 1997). Furthermore, direct applications of insecticides to cattle provide only marginal control, especially for animals in pasture settings (Marcon et al. 1997, Campbell et al. 2001). Cultural practices involving the removal and dispersal of substrates can serve as a useful tool for reducing breeding sites, but are tedious and costly (Zumpt 1973) suggested that spraying cattle with repellents or applying contact insecticides to fly resting areas would suppress the development of stable fly infestation more effectively

than direct insecticide application. Repellency might result from olfactory or contact stimulation that causes flies to avoid treated areas and substrates.

The use of repellents is considered as one of the most effective tools for protecting humans from biting insects (Curtis et al. 1991, Barnard 2000, Isman 2006). Recent studies have further demonstrated the effectiveness of botanical based repellents as alternatives against dipteran blood-sucking insects, particularly mosquitoes (Barnard 1999, Sukumar et al. 1999, Schultz et al. 2004, Zhu et al. 2006). The use of repellents may represent an effective alternative strategy for reducing the impact of flies on livestock. Unfortunately, with the exception of certain insecticides that may also act as partial repellents, such as the organophosphates used in insecticide impregnated ear tags (Liddel and Clayton 1982, Hogsette and Ruff 1986, Harris et al. 1987), few repellents have been made commercially available. However, the effectiveness of such ear tags for controlling stable flies is limited because the tags do not provide adequate coverage of the lower portions of the legs of cattle, on which stable flies commonly feed (Foil and Hogsette 1994). Furthermore, organophosphate-impregnated ear tags have very little effect on stable fly feeding. The tags were originally developed for horn fly control and stable flies may be less susceptible or may not remain on the host long enough to receive a toxic dose (Guglielmone et al. 2004). Not surprisingly, there is considerable interest in developing botanical repellents because of increasing regulation and negative public perception of synthetic insecticides (Coats 1994, Isman 2006). In addition, the U.S. Environmental Protection Agency (EPA) has ruled that many essential oils of plant origin are exempt from regulation under the Federal Insecticide, Fungicide, and Rodenticide Act of 1996. This shields these alternative repellent oils and compounds from prohibitive

registration costs. The development of botanical repellent compounds would be a valuable tool in the integrated management of a range of biting flies that transmit livestock and human diseases. Zhu et al. (2009, 2010) reported that catnip oil at a dosage of 20 mg effectively deterred the blood feeding of stable flies (>95%) in a laboratory bioassay, and a wax-formulated catnip oil applied in stable fly resting areas was able to repel flies for up to 3 h. Hieu et al. (2010) have further shown that essential oil of patchouli, *Pogostemon cablin* (Blanco) Benth, can prevent stable flies from biting humans for up to 3.7 h.

The present paper reports our findings on: (a) the feeding and oviposition repellency of the essential oil of catnip, *Nepeta cataria* L., including its major ingredient compounds [(*Z,E*)-nepetalactone and (*E,Z*)-nepetalactone], against stable flies; (b) comparisons of the effectiveness of the feeding repellency of catnip oil with those of previously identified biting insect repellents; (c) the effectiveness and longevity of catnip oil formulations that repel stable fly attack on cattle in the field, and (d) the effectiveness of catnip oil as an oviposition repellent against stable flies.

MATERIALS AND METHODS

Repellent candidates

Three plant essential oils were tested for feeding repellency of stable flies. Sandalwood oil (*Santalum album*) and amyris oil (*Amyris balsamifera* L.) were purchased from Olympian Labs, Inc. (Scottsdale, AZ, U.S.A.) and Sigma-Aldrich, Inc. (St Louis, MO, U.S.A.), respectively. Catnip essential oil was purchased from Bramble Berry, Inc. (Bellingham, WA, U.S.A.). The oil chemical composition was determined by

gas chromatography-mass spectrometry (GC-MS) analysis based on methods described in Schultz et al. (2004) and Zhu et al. (2006), which showed it to comprise (*Z,E*)- and (*E,Z*)-nepetalactone (80%) and caryophyllene (18%). The two nepetalactones were accumulated from the purchased catnip essential oil and purified (>95%) following the method described in Peterson (2001). *N,N*-diethyl-3-methylbenzamide (DEET) was purchased from Morflex, Inc. (Greensboro, NC, U.S.A.) with >98% purity. (–)-Isolongifolenone (J4- 118) was prepared from (–)-isolongifolene purchased from Sigma-Aldrich, Inc. as described in Wang & Zhang (2008) with >98% purity. The 2-methylpiperidinyl-3-cyclohexen-1- carboxamide (AI3-37220) was also purchased from Morflex, Inc. as a mixture of four diastereoisomers. Optically pure diastereoisomer (1*S*,2*S*)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide (SS220) was purchased from Sai Dru Syn Laboratories Ltd (Hyderabad, India) (95% stereoisomeric and >99% chemical purity).

Insects

Stable flies used for laboratory bioassays were sourced from colonies maintained at the U.S. Department of Agriculture, Agricultural Research Service, Agroecosystem Management Research Unit (Lincoln, NE, U.S.A.). The flies were maintained at 23 ± 2 °C at variable relative humidity (RH) of 30–50% and an LD 12 : 12 h photoperiod. Adults were fed on citrated bovine blood (3.7 g sodium citrate/L) in a blood soaked absorbent pad (Stayfree®; McNeil-PPC, Inc., Skillman, NJ, U.S.A.) placed on top of a screened cage.

Feeding repellency assay

The laboratory bioassay for testing feeding repellency used six-well feeding reservoirs similar to the *in vitro* Klun and Debboun system described by Klun et al. (2005), but modified for stable fly use (Zhu et al. 2009). Newly emerged adult stable flies were supplied with 10% sugar water on day 1. The sugar water was then removed and flies were fed with bovine blood once or twice. Adults (aged 2–3 days) were starved for 48 h prior to each test. The plant essential oils (20 mg) and synthetic catnip constituent compounds at three doses (0.2 mg, 2 mg, 20 mg) were weighed out. Each of them was dissolved in 300 μ L of high-purity solvent (hexane) (Honeywell, Burdick & Jackson, Inc., Muskegon, MI, U.S.A.) and then evenly applied to an outer layer cut from a sanitary pad (4 \times 5 cm). When the solvent had evaporated (after 2–3 min), the repellent-impregnated layer was placed on top of the blood-soaked sanitary pad in the reservoir well. Starved stable flies were transferred into each of the six testing cells (average of three to five flies in each cell). After 4 h, surviving stable flies were anaesthetized with carbon dioxide (CO₂) and checked for feeding status by squashing their abdomen to determine the presence of blood. Flies in the repellent bioassay were exposed to randomized treatments (essential oil repellent candidates and various dosages of catnip oil) until at least six to eight replicates had been completed (new groups of flies were used for all replicated experiments). In tests of the feeding repellency of several newly identified synthetic insect antifeedants/repellents and DEET, dosages of 20 mg were used and treatments were repeated at least five times.

Oviposition repellency

A total of three oviposition repellency experiments on the effects of catnip oil, its active constituent compounds and its spatial repellency on the oviposition of gravid female stable flies were conducted. A two-choice oviposition repellency assay was performed in a screen cage measuring $0.4 \times 0.4 \times 0.4$ m with ~400 mixed-sex stable flies held in the laboratory at 23 ± 2 °C and 30–50% RH. The stable flies were 10 days old and >90% of the females were gravid (10 randomly selected females were dissected to determine the stage of ovarian development). These flies had not been previously provided with oviposition substrates. Oviposition jars were prepared by placing one end of a black cloth (10×30 cm) in a small glass jar (5.1 cm diameter, 7.6 cm high) filled with ~200 mL of water to act as a wick. The middle portion of the cloth was draped over the mouth of the jar and fastened in place with a rubber band. The remaining end of the cloth was folded back to form a tunnel over the top of the jar for oviposition. Two oviposition jars were provided; the area of the black cloth forming the tunnel to one jar was treated with 100 mg of catnip oil in 1 mL of hexane (treatment jar), whereas the cloth forming the tunnel to the other jar was treated with 1 mL of hexane only (control jar). The two jars were set in opposite corners 40 cm apart and their positions were alternated among replications to minimize possible position effects. After 6 h, the jars were removed from the cage and eggs laid were rinsed from the black cloth with water into a plastic pan ($20 \times 10 \times 5$ cm). The eggs were transferred to a 10-mL graduated cylinder and the number of eggs was estimated ($1 \text{ mL} \approx 8000$ eggs). This bioassay was replicated 11 times.

In the second experiment, a total of four oviposition jars (four-choice test) were randomly placed 30 cm apart in the corners of the screen cage. The tunnel areas of three jars were treated with randomly selected repellent candidates [100 mg of catnip essential oil, 100 mg of (*Z,E*)-nepetalactone, 100 mg of (*E,Z*)-nepetalactone] in 1 mL of hexane topically applied to folded layers of the black cloth. The cloth (tunnel area) of the fourth jar was treated with 1 mL hexane as a control. The methods described above were used to measure the number of eggs laid in the various treatments. The experiments were replicated six times.

A third assay for spatial oviposition repellency was conducted within a large screen cage ($1.0 \times 0.5 \times 0.5$ m) inside a greenhouse at a temperature of 27 ± 5 °C between 10.00 hours and 16.00 hours. Approximately 500 gravid female stable flies were released into the screened cage. Inside the cage, one catnip-treated oviposition jar and another untreated jar were placed 70 cm apart. Rather than topically applying repellent onto the tunnel area of the oviposition jar as in the previous experiments, we impregnated the upper one-third of four Whatman No. 1 filter papers (Whatman International Ltd, Maidstone, U.K.) used as a barrier with 100 mg of catnip oil (in 1 mL hexane) so that the treated areas extended about 2 cm above the oviposition jar. The centered oviposition jar was placed at a distance of 2 cm from the repellent barrier. Experiment duration and egg counting methods were as described above. The experiments were repeated 10 times.

Repellency of catnip oil formulations against stable flies in the field

Two types of catnip formulation were prepared for evaluating catnip oil repellency in field trials. The oil-based formulation was prepared by adding 15% pure catnip essential oil (Bramble Berry, Inc.) to mineral oil (light oil form). For the

water-based formulation, Triton X-100 (Sigma-Aldrich, Inc.) was used as a surfactant. This allowed the catnip oil to disperse in the water with minor agitation and remain in emulsion. The catnip water-based formulation contained 30% catnip oil, 67% water and 3% Triton X-100. The repellency against stable flies of two catnip formulations was tested on heifers and steers under field conditions during the summers of 2009 and 2010. The repellency tests were carried out in Lincoln/Mead (Agricultural Research and Development Center) and North Platte (University of Nebraska, West Central Research and Extension Center), Nebraska. Tests were conducted using criteria specified by the American Society for Testing and Materials (ASTM, 1980) and protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska (IACUC protocol no. 06-12-053C). To test the effectiveness of the catnip oil-based formulation, one front and one rear leg (randomly selected) were treated with 15% catnip oil (~25 mL of repellent formulation was applied per animal leg) using a bath sponge (Walmart, Inc.) soaked in formulation. The remaining two legs were similarly treated with mineral oil only as a control. The number of stable flies on each leg was visually counted hourly between 10.00 hours and 18.00 hours. These counts were confirmed using Microsoft Image Viewer to examine photographs taken during the observations. These tests were repeated three times, using five animals in each test. At least 2 weeks elapsed between tests to ensure that no residue of repellents applied in prior experiments remained.

For applications of the water-based catnip formulation, we used a compressed-air hand gun (J. E. Adams Industries Ltd, Cedar Rapids, IA, U.S.A.) powered with air pressure at 241 KPa. The test cattle ($n = 4$) were restrained in a cattle chute and a total of

250 mL of water-based catnip formulation was evenly sprayed on all four legs and the lower body of each animal. Control cattle ($n = 4$) were treated with the same volume of formulation, but only mineral oil had been added (30% of total volume). Catnip-treated cattle were placed in pens situated at least 50 m downwind of the pens containing control animals. Fly densities on both treatment and control cattle were observed to be similar (10–15 flies per leg) before the tests. The method used to count stable flies on each animal was similar to those described for oil formulations. Observations were made hourly for a total of 6 h (12.00–18.00 hours) and again at 24 h after application.

Statistical analysis

Percentiles of feeding repellency [(number of flies feeding on control cattle - number of flies feeding on treated cattle)/ number of flies feeding on control cattle \times 100] were determined and transformed to arcsine square-root values for analyses of variance (anova) using IBM spss Statistics 18 (SPSS, Inc., Chicago, IL, U.S.A.). Significant differences at $P = 0.05$ among percentage means were determined using Duncan's test (when the number of replicates per treatment were equal) and the Student–Newman–Keuls test (when these numbers were not equal). Repellencies are reported before arcsine transformation.

The number of eggs laid in the four-choice assay was subjected to anova and the treatment means were separated using Duncan's multiple range test, with $\alpha = 0.05$. Student's t -test was used to compare differences in egg counts in the two-choice and the spatial oviposition repellency assays. Comparisons between treated and control animals of numbers of flies observed were performed using repeated-measures anova. Results with P -values of <0.05 were considered statistically significant.

RESULTS

Stable fly feeding repellency assay

The feeding repellencies of three essential oils (catnip, sandalwood, amyris) were evaluated using a laboratory bioassay newly developed for biting flies. Catnip oil strongly repelled stable flies from blood feeding with a repellency rate of 98%, which was significantly higher than those of the other plant essential oils tested ($F = 30.16$, d.f. = 3,28, $P < 0.05$) (Fig. 1A). Over 98% of control flies were observed to be blood fed. An additional dose–response test of catnip oil revealed that the highest dosage of 20 mg provided better protection than the two lower doses tested ($F = 12.01$, d.f. = 4,32, $P < 0.05$) (Fig. 1B). By itself, mineral oil (light viscosity) was also found to deter feeding (~30%) at a rate comparable with that of the lowest concentration of catnip oil (0.2 mg). The (Z,E)- and (E,Z)-nepetalactones prevented stable flies from blood feeding as effectively as catnip essential oil ($F = 57.15$, d.f. = 4,35, $P < 0.05$) (Fig. 1C). Significantly lower repellency (<20%) was observed for caryophyllene. We further compared catnip oil with several recently identified deterrents and repellents for repellency against blood-feeding arthropods. Substances tested included (–)-isolongifolenone (J4-118), 2-methylpiperidinyl-3-cyclohexen-1-carboxamide (AI3-37220) and (1S,2_S)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide (SS220), as well as the most commonly used mosquito repellent, *N,N*-diethyl-3-methylbenzamide (DEET) (Klun et al. 2003, 2004, Zhang et al. 2009). The repellencies of catnip oil and AI3-37220 were significantly higher than that of DEET ($F = 11.41$, d.f. = 5,35, $P < 0.05$) (Fig. 2). No differences were observed among DEET, J4-118 and SS220 in preventing stable fly feeding.

Oviposition repellency of catnip oil

When gravid female stable flies were given a choice of oviposition site, they laid approximately 20 000 eggs on the control site and ~100 eggs on the catnip-treated oviposition medium (Fig. 3A) ($t = 2.18$, $P < 0.05$). The oviposition repellency of catnip was $97.26 \pm 0.79\%$. A further four-choice oviposition experiment to compare the repellency of catnip oil with that of its major constituent compounds (nepetalactones) revealed that significantly more eggs were laid in the control jars ($F = 83.36$, d.f. = 3,16, $P < 0.05$), but no differences were found in numbers laid in the catnip- and nepetalactone treated jars (Fig. 3B). We also observed that female stable flies laid fewer eggs in oviposition jars surrounded by the catnip oil-treated barrier, suggesting that catnip oil may act as an olfactory spatial repellent. Results of the oviposition repellency experiment using the catnip-treated barrier that surrounded but was not directly applied to the oviposition medium showed that gravid females laid <100 eggs in catnip-treated jars, whereas 14 345 eggs were found in the control jars ($t = 2.26$, $P < 0.05$) (Fig. 3C). The spatial oviposition repellency of catnip was estimated at $98.43 \pm 0.52\%$.

Field repellency test

Numbers of stable flies on cattle legs treated with the 15% catnip oil-based formulation were significantly lower than those on untreated legs (Fig. 4A). Effective repellency (>90%) lasted up to 6 h after application ($P < 0.05$) and disappeared in the seventh hour after application. Effective repellency (>90%) of the water-based catnip formulation (30%) was observed for only 4–5 h after application ($P < 0.05$) (Fig. 4B).

DISCUSSION

Catnip has been reported as a potential alternative insect repellent, for which repellency against several disease-transmitting urban insect pests, including mosquitoes and cockroaches, has been documented (Peterson 2001, Schultz et al. 2004, Bernier et al. 2005, Zhu et al 2006). As a folk remedy, catnip has also been reported to repel up to 13 families of insects (Eisner 1964). Catnip has very low acute oral, dermal and inhalation mammalian toxicities, levels of which are similar to those of other EPA-approved mosquito repellents (Zhu et al. 2009). The current study shows catnip oil to be a relatively strong feeding and oviposition repellent against stable flies. Our observations in laboratory spatial oviposition assays and field animal tests further suggest that catnip also serves as a spatial repellent to stable flies. This is the first study to demonstrate that catnip oil can inhibit gravid stable fly egg-laying behaviour (98% inhibition). Therefore, it may be developed to control stable fly oviposition and, together with other effective means (such as the push–pull strategy), to further reduce populations of this pest in the field.

Sesquiterpene-rich amyris and sandalwood plant essential oils are known to provide significant repellency against various arthropod species, particularly against several mosquito species (Paluch et al. 2009). In comparison with catnip oil, their repellency against stable fly biting is relatively low. Similarly, the most common personal protectant against biting insects, DEET, was not found to be a strong repellent against stable flies (repellency rate: 50%). Schreck et al. (1978) reported that DEET applied at a dose of 250 mg on the forearm deterred stable fly biting for only 2–3 h. (–)-Isolongifolenone, 2-methylpiperidinyl-3- cyclohexen-1-carboxamide and (1S,2_S)-2-

methylnonyl-3-cyclohexen-1-carboxamide are three recently identified repellents against mosquitoes and ticks (Klun et al. 2001, 2003, 2004, Zhang et al. 2009). The levels of repellency of these compounds against stable flies were similar to that provided by catnip oil. Catnip oil has been reported as an effective contact repellent against several mosquito species, but its repellency varies with dose and species (Bernier et al. 2005). Our study suggests that catnip oil must be administered at a dosage of ≥ 20 mg (1 mg/cm²) to provide effective repellency (>90%) against stable flies. Thus, using catnip oil as a topical repellent may be costly. From our observations in feeding and oviposition repellency assays in the laboratory and in cattle in the field condition, we noted that stable flies avoid the catnip treated substrates and hosts by abruptly flying away (2–3 cm from catnip-treated targets). Our indoor behavioural assay conducted in a single caged olfactometer further suggests that catnip oil may act as a spatial repellent (Zhu et al. 2010). Electroantennogram (EAG) trials of stable fly antennae exposed to catnip volatiles also elicited a significant response (Zhu et al. 2010), with a unique EAG pattern of both positive and negative peaks. Similar EAG responses in other insects may reflect the electrophysiological nature of insect response to repellent compounds (Contreras et al. 1989, Pavis and Renou 1990, Jyothi et al. 2008).

Most insect repellents operate in the vapor phase, in which volatiles can be detected by insect olfactory sensilla and are then kept at a distance (Garson and Winnike 1968). Repellents with high vapor pressure, such as catnip oil, may offer protection at low concentrations, but this carries a risk for the loss of repellency within a short time. This was confirmed in our field repellent trials, in which both formulations of catnip oil

provided only about 5–6 h of protection. The longevity of a repellent formulation may not relate to its concentration, but, rather, to the physical and chemical properties of the repellent compound. This is supported by the fact that the 30% water-based formulation resulted in shorter effective time than the 15% oil-based formulation. More work is necessary to discover and develop more efficient formulations of plant essential oils in order to extend their longevity as repellents in general.

In conclusion, catnip oil and its major constituent compounds, nepetalactones, act not only as effective feeding and oviposition repellents, but also have a strong spatial repellency. The *in vitro* blood-feeding assay has proven a useful screening tool for discovering novel repellents for stable flies. Field trials conducted on cattle of two catnip oil formulations found these gave 5–6 h of protection against stable flies. Formulating catnip oil to meet USDA organic standards may also have promise as a method for stable fly control in organic dairy farms (Isman 2006, 2008).

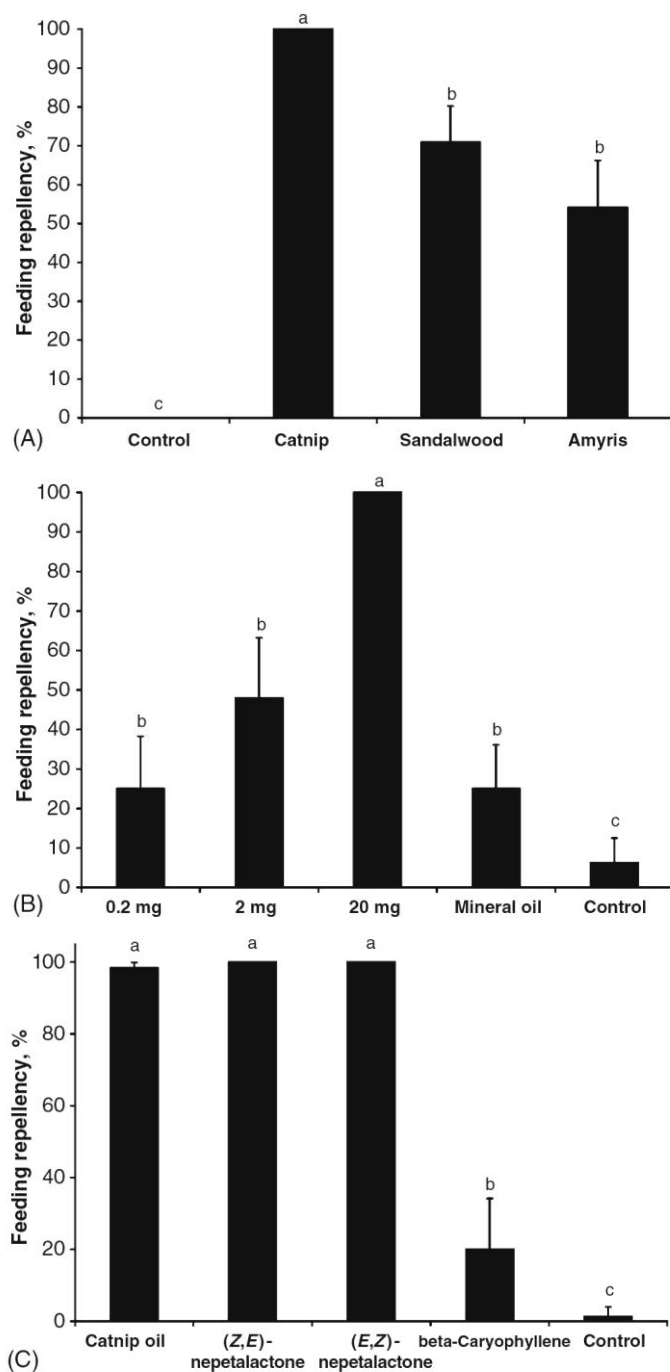


Figure 1 Mean percentage of feeding repellency observed from starved stable flies treated with A). three different plant essential oils and the control ($N=8$); B). Three different dosages of catnip oil and the controls ($N=6-8$); C). 20 mg of catnip oil and its synthetic ingredient compounds ($N=8$) in laboratory *in vitro* system. Means with different letters are significantly different at ($P < 0.05$, ANOVA followed by Duncan's test (A and C) and Student-Newman-Keuls test (B), error bars show standard errors of the mean.

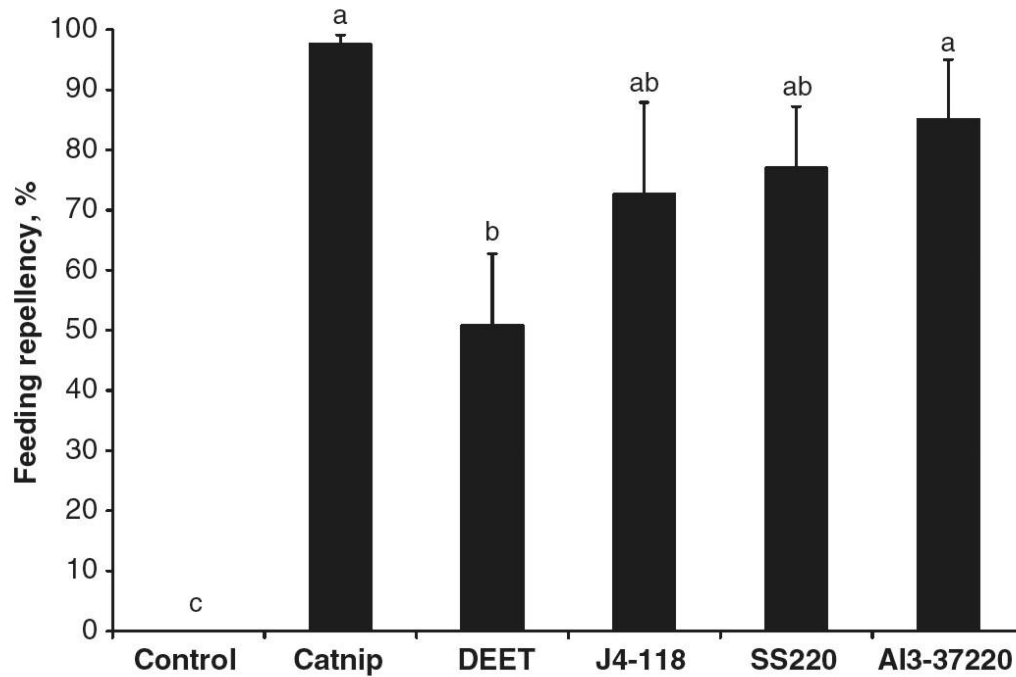


Figure 2 Comparisons of feeding repency observed from starved stable flies to catnip oil (20mg) and other recently-identified insect repellents at the same dosage in laboratory *in vitro* system ($N=5-8$). Means with different letters on top of bars are significantly different ($P < 0.05$, ANOVA followed by Student-Newman-Keuls test), error bars show standard errors of the mean

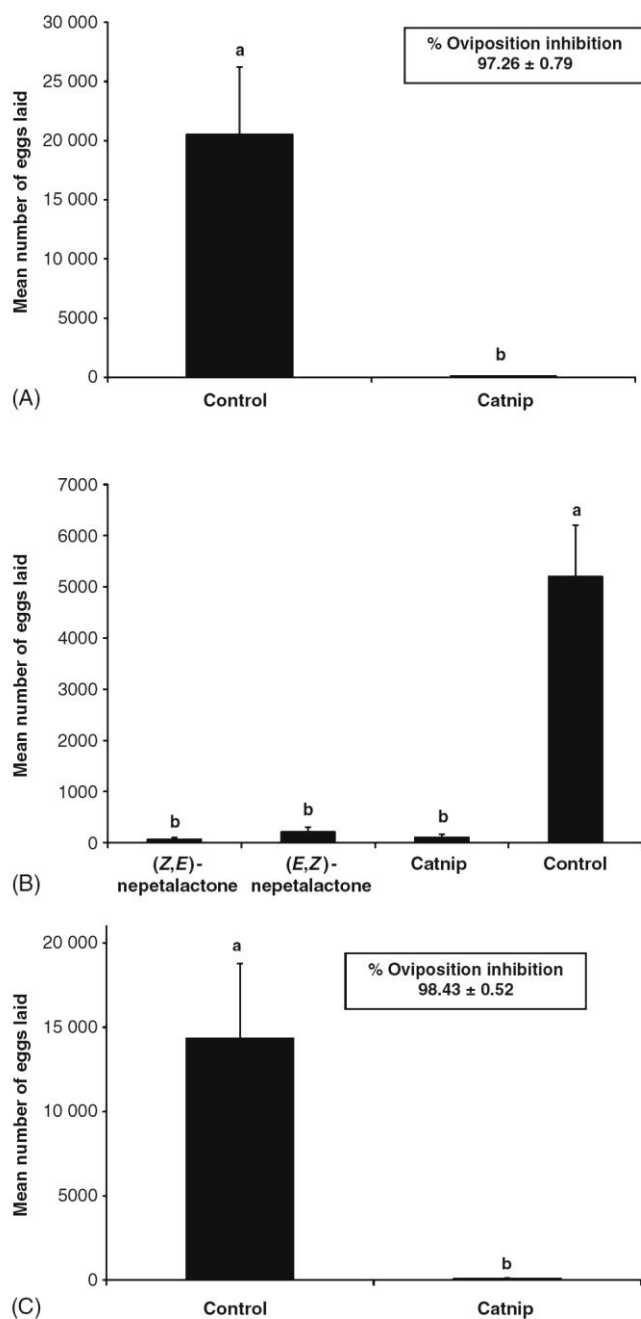


Figure 3 Mean number of eggs laid from oviposition jars treated with A). 100 mg of catnip oil, or without ($N=11$); B). 100 mg of catnip oil, two catnip components and the control ($N=5$); C). mean numbers of egg laid from oviposition jars surrounded by a catnip oil-treated barrier, or without ($N=10$). Means with different letters (A and C) above the bars are significantly different at $P < 0.05$ level (Student *T*-test). Means with different letters (B) on top of the bars are significantly different at ($P < 0.05$, ANOVA followed by Duncan's test). Error bars show standard errors of the mean.

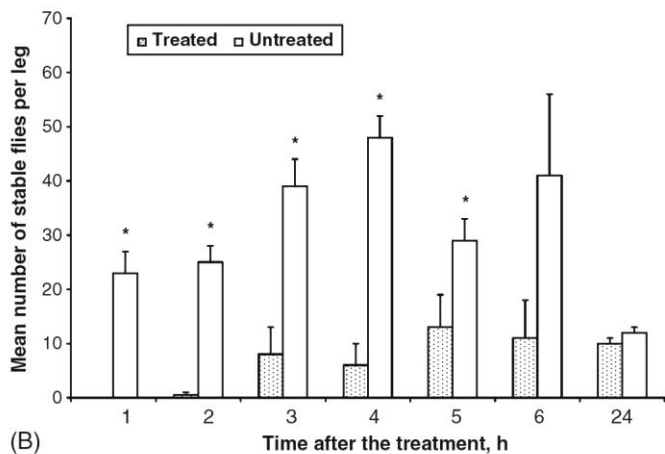
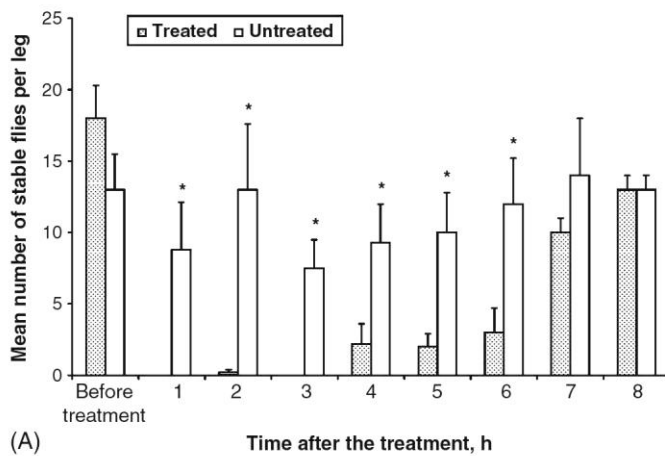


Figure 4 Mean number of adult stable flies observed landing on legs of cattle treated with A). 15% of catnip oil-based formulation and the control; and B). 30% of water-based formulation and the control. Means with an asterisk above a pair of bars by time after treatment are significantly different at ($P < 0.05$, Student *T*-test). Error bars show standard errors of the mean.

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Chapter IV Behavioral response of stable flies, *Stomoxys calcitrans* (L). (Diptera: Muscidae) to host associated volatiles

ABSTRACT Stable flies (*Stomoxys calcitrans*) are blood-feeding synanthropic pests that can produce significant economic losses in livestock through dramatic weight loss. Earlier studies have shown that stable fly antennae contain olfactory sensilla that respond to host and host environment associated odors. Field observation showed that the abundance of stable flies significantly increased in areas sprayed with cattle manure slush. Volatile compounds of manure slush were identified, and stable fly behavior was investigated in both laboratory bioassays and field trapping experiments. Result from the single cage olfactometer assays revealed that 1-octen-3-ol, and the combination of phenol and *p*-cresol or *m*-cresol was attractive to adult stable flies. The combination of phenol (100 µg) and *m*-cresol (4 µg) was the most attractive to stable flies among the tested blends and the single compound. In the field, alsynite traps baited with these same compounds caught significantly more stable flies than those without attractant lures. The highest dosages of both 1-octen-3-ol and the combination of phenol and *p*-cresol were the most attractive to stable flies in both lab bioassays and field trapping tests.

Key words Stable fly, *Stomoxys calcitrans*, olfactometer, synergistic

This research will be submitted to the Journal of Medical and Veterinary Entomology.

INTRODUCTION

Stable flies, *Stomoxys calcitrans* (L) are biting blood-feeding flies that can cause significant weight loss in cattle (Campbell et al. 2001, Catangui et al. 1997) and reduce milk yields (Mullens et al. 2006). They are considered the most important arthropod pest of livestock with losses estimated to exceed \$2 billion per year in the United States (Taylor et al. 2012). Similar to other biting flies, *S. calcitrans* host searching behavior is partially mediated by host and host environment related odors (Gatehouse and Lewis 1973, Warnes and Finlayson 1958a, b, Holloway and Phelps 1991).

Despite of the discovery of potent attractants for the tsetse fly in the 1980s, research on visual ecology (Gibson and Torr 1999) have further set the stage for the development of cost-effective bait technologies for tsetse fly control (Vale and Torr 2004). Similar to tsetse flies, stable flies are also obligate blood-sucking flies. They share the common behavior, such as feeding mostly in day light, but stable fly adults prefer sunlight, and will also follow animals into buildings to feed. They typically bite during morning or late afternoon. Usually stable flies are inactive at night. Based on similar blood-feeding and host-seeking behaviors, tsetse fly bait technologies may be applicable to control stable flies as well. However, this approach may be practical only if the visual cues that attract flies to traps or targets can be further enhanced (Green 1994, Hall and Wall 2004) and if economical baits can be developed to improve the range and efficiency of attraction.

In 1999, Gibson and Torr conducted a study investigating the visual and olfactory responses of haematophagous Diptera to host stimuli, including activation and ranging behavior, long-rang and short-range olfactory responses and visual responses. They found

that biotic and environmental constraints resulted in unique host seeking behaviors in different species of biting dipterans that are mainly relied on their olfactory and visual stimuli. Other researchers have also reported that attractant odor compounds are used for host seeking by several biting flies (Hayes et al. 1993, Djitey et al. 1998, Nilssen 1998, Cilek 1999, Mohamed-Ahmed and Mihok 1999, Kristensen and Sommer 2000, International Atomic Energy Agency (IAEA) 2003, Birkett et al. 2004, Krāmar et al. 2005). Recent studies which have focused on the attractiveness of different host associated volatiles revealed candidate attractants which evoked stable fly responses (Jeanbourquin 2006, Tangtrakulwanich et al. 2011). Both studies have demonstrated electroantennogram (EAG) responses elicited from stable fly's antennae in both sexes. They have further reported the highest electroantennogram (EAG) responses of both female and male stable fly antenna were elicited from 1-octen-3-ol, indole, phenol and *p*-cresol.

Host derived volatiles, such as 1-octen-3-ol (octenol), phenol, indole and *p*-cresol, have been identified from aged urine and manure of bovines. They have been demonstrated to be extremely attractive to various biting flies (IAEA 2003). Baits with these attractants have been reported to increase the trapping efficacy for controlling of stable flies (Schofield et al. 1995, Warnes and Finlayson 1985). From a survey study on monitoring the field population of stable flies in Nebraska, USDA-AMRU observed that extremely high numbers of stable flies were caught from traps placed close to the areas sprayed with the cattle manure slush. This indicates that volatiles released from the cattle manure slush may play critical roles for this enhanced attraction. Additional research is needed to identify these potential attractant compounds that can be further used to

enhance catches of stable flies with baited traps (Williams 1973, Meifert et al. 1978, Ruff 1979, Gersabeck et al. 1982, Gersabeck and Merritt 1985, Hogsette 1984, Pickens and Hayes 1984).

The objectives of this study were 1) to characterize volatile profiles of the cattle manure slush attractive to stable flies, and their behavior responses to some selected identified attractant compounds, 2) to demonstrate their synergism in trap catches under the field conditions.

MATERIAL AND METHODS

Fresh cow manure volatile collection and analysis

Cattle manure slush (a runoff of manure/urine mixed with water and accumulated organic waste in feed lots) was collected (< 24 h old) from cattle production pens at the University of Nebraska Agricultural Research and Development Center, Ithaca, NE. The manure was placed in 50 ml Corning screw cap conical bottom centrifuge tubes (#430290, Corning®) and transported to the laboratory. Ten milliliter of the slush was transferred to a glass vial (20 ml Micro Liter Analytical Sample Vials) sealed with the parafilm (Pechiney Plastic Packaging PM992-Parafilm M®). Solid-phase microextraction (SPME) was used for volatile collection. The SPME fibers (75 µm PDMS/Carboxen, Supelco, Sigma-Alrich®, Saint Louis, Missouri) were pre-conditioned for 6 minutes in an inlet of the gas chromatograph (GC) at 300 °C with a continuous helium stream before the collection. SPME fibers were inserted through the parafilm into the collection vial at 1-2 cm above the slush, and allowed to absorb volatiles at 10, 20, or 30 seconds per time interval. After the completion of collecting, SPME fibers were then injected for analyses in Agilent 6890 GC with a 5973 Mass spectrometry (Agilent Technologies, Palo Alto,

CA). SPME fibers were thermo-desorbed at 300°C and analyzed through either a DB-Wax or FFAP column (30m x 0.25 mm diameter, with 0.25µm film thickness, J&W Scientific, Agilent). Helium at a flow rate maintained at 1.5 mL/min was used as the carrier gas. Samples were injected under the splitless mode. The temperature program for the GC analyses was 50°C for 3 min, rising by 10°C/min to 240°C. Compounds were identified by comparisons of retention times and mass spectra with those of synthetic standards using Wiley MS Library Database (Agilent). A total of 6 collections were conducted.

Single Cage Olfactometer Trials

Two choice bioassays were conducted using a single cage, dual port olfactometer (see photo below) designed to measure upwind attraction of stable flies to the cow manure slush. Stable flies were from 4 years old colonies (United States Department of Agriculture Research Service, Agroecosystem Management Research Unit, at Lincoln, NE) maintained at $23\pm 2^\circ\text{C}$ with variable humidity (30 to 50%) and a 12L:12D photoperiod. Adults were fed citrated bovine blood (3.7 g sodium citrate/litter) in



a blood soaked absorbent pad (Stayfree[®], McNeil-PPC Inc., Skillman, New Jersey). Flies were 3 to 4 days old and starved for 24 h prior to initiation of the bioassay.

The olfactometer (Posey et al. 1998 as modified by Zhu et al. 2010) was constructed of clear, 4 mm thick glass (Pyrex[®]), had overall dimensions of 96 cm x 50 cm x 25 cm, and was placed on a white table. The design consisted of a central rectangular glass box with two collection ports (30 cm long glass tubes, 10 cm apart) located at one end of the box. A similar single introduction port was located at opposite end of the box. The ends of all ports were covered with gauze mesh fabric (1.0 mm). Two small fans, situated in front of each of the collection ports and regulated by separate voltage regulators, generated wind speeds in the ports of 0.35 to 0.4 m/s. Calibration trials detected no fly bias for either of the collection ports.

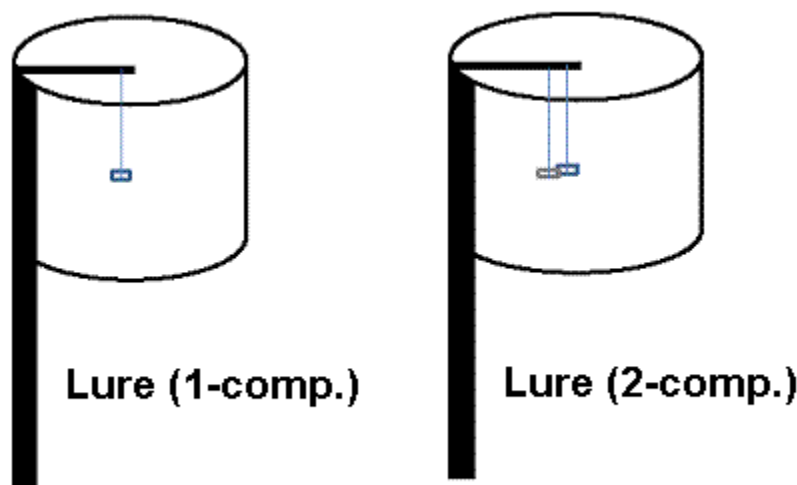
Volatiles were tested as single compounds or as mixtures. Solutions of single compounds (phenol, *p*-cresol, *m*-cresol, 1-octen-3-ol) and mixtures (phenol+ *m*-cresol or phenol+ *p*-cresol) were prepared in hexane at three dosages, 1, 10 and 100 µg/µl. Controls consisted of hexane only. For testing, 10 µl of one of the testing solutions were applied to a small triangle filter paper that attached to a metal wire, and placed 2 cms away from the end of the collection port. The control was the same way as the treatment, except only 10 µl of hexane applied. A magnet, set on the outside of the glass collection port, was used to secure triangles pierced with an insect pin 3 cm down inside the port. Each test consisted of a series of 4 runs with a single fly introduced to a random sequence of each of the test volatiles. The fly would be released in the introduction port and observed for up to 5 minutes (recorded as non-response) it was then removed, and introduced with a new fly. When a fly entered either one of the collection ports, the

behavioral choice was scored and the run stopped. For subsequent runs, the location of the control and treatment was alternated from one collection port to the other. This process was continued until 4 flies were tested, then the systems were cleaned. The olfactometer was cleaned with acetone and then 70% ethanol. A total of 180-200 flies were tested for each set of test volatiles. Testing of each volatile was repeated 3 to 4 times (n=100 to 200). There were 46.3% of the total flies that did not response to either treatment port or control port.

Results (percentage of flies entering per port) were analyzed using Student's t-test after data was checked for homogeneity of variance and normality of data. Values of $P < 0.05$ were considered significant. All analyses were conducted using SAS, version 9.1 (SAS Institute 2004).

Field Experiments

Experiments conducted from June to September 2010 evaluating the attraction of selected volatiles to stable flies in the field. Traps consisted of a cylindrical alsynite panel 30.5 cm in height and 28 cm in diameter similar to Broce traps described by Taylor and Berkebile (2006). The panels were covered with 10-mil Sur-Flex plastic sleeves (Flex-o-glass, Inc., Chicago, IL) coated with Tangle-Trap (The Tanglefoot Co., Grand Rapids, MI) diluted 1:1 with low-odor paint thinner (Sunnyside Corp., Wheeling, IL). Attractant lures tested were placed as described in the figure below.



Experiments 1 A balanced incomplete block design with 7 treatments were randomly assigned to traps. Four replicates were tested in 7 areas at the University of Nebraska, East campus, Lincoln, NE. All traps were placed approximately 100 meters from cattle pens. Treatments were 1 or 100 μg of test compounds in 2000 μl of hexane. The solutions were then applied to a 1.27 cm length of cotton roll and hung from the top of the trap by a 15 cm stainless steel wire (above sketches). The attractant chemicals were from Sigma-Aldrich (St. Louis, MO, U.S.A.) with > 98% purity. Four Broce traps were placed randomly 20 m apart in each area. The numbers of stable flies captured in each trap were counted on days 1, 3 and 5. The experiment was repeated 3 times.

Experiment 2 Based on results from experiment 1, the dose responses of 1-octen-3-ol and phenol were further tested at concentrations of 100, 500, and 2500 μg of test compounds in 2000 μl of hexane similar as described above. Application of the lures to the traps was similar to experiment 1. Testing was conducted at the four sites (where the highest numbers of stable flies were collected) used in experiment 1. The design was a

randomized complete block (RCBD) with 4 replications. The experiment was repeated 3 times. Stable flies were collected on days 1 and 2.

Experiment 3 Based on the results from the olfactometer assay and field experiment 2, I tested mixtures of phenol (2500 μ g) and *m*-cresol (100 μ g) and phenol (2500 μ g) and *p*-cresol (100 μ g) and only phenol (2500 μ g). Only the test concentrations differed but attractant solutions were prepared as described in experiment 1. The combinations of the two compounds were installed by hanging the cotton roll soaked with first compound (according to the assigned concentrations) to the trap as described for previous experiments. The second cotton roll was then soaked with the other compound and hung about 0.5 cm apart from the first cotton roll (See the sketch in previous page). The experiment was arranged in a RCBD designs with 5 replications. Data were collected on days 1 and 2. The experiment was repeated 3 times.

Data analysis for all field experiments

Factorial analysis of variance (ANOVA) was used to analyze trap catches (number of flies collected per trap) in all field tests. Means were compared using least square difference (LSD) test. Values of $P < 0.05$ were considered significant. Analyses were conducted using SAS, version 9.1 (SAS Institute 2004).

RESULTS

Field stable fly population monitoring experiments showed that significantly fly catches appeared after fields being sprayed with cattle manure slush (Figure 1). Gas chromatography analyses of SPME collection from field collected cattle manure slush revealed eight volatile compounds detected (Figure 2). By comparing retention times and

mass spectra to those of synthetic standards, they were identified as acetic acid, propanoic acid, butanoic acid, phenol, *p*-cresol, *m*-cresol, 4-ethylphenol and indole.

The attraction of the identified phenol, *m*-cresol or *p*-cresol and 1-octen-3-ol (one of the previously reported aged cow urine compounds), were tested in a single cage olfactometer. At a lower dosage (1 µg), significantly more stable flies responded to all four selected compounds, compared to the control (Figure 3). Inhibition was further noticed from tests conducted at 10 µg dosage for *m*-cresol or *p*-cresol. More stable flies were found entering the ports with 10 µg of phenol and 100 µg of 1-octen-3-ol. The treatments with mixtures containing phenol (100 µg) and 4 µg of *m*-cresol or *p*-cresol were more attractive to stable flies, relative to the controls (Figure 4).

A series of three field trails were conducted to test stable fly attraction to Broce traps baited with individual attractants (1-octen-3-ol and phenol) and combinations of all attractants and at various concentrations. The effectiveness of lure at different ages was also compared.

In the first experiment, the low dose (1 µg) (1-octen-3-ol, phenol, and a mixture of 1:1) did not differ significantly in attracting more stable flies than the control, for any test day (Figure 5). At the high dose (100µg) of both 1-octen-3-ol and phenol attracted significantly more stable flies than the mixture and the control on the 1st day and 3rd day (Figure 5, $F_{\text{day-1}} = 3.03$, $d.f. = 3,24$, $P < 0.05$; $F_{\text{day-3}} = 3.45$, $d.f. = 3,24$, $P < 0.05$). However, the efficacy of the all attractants significantly decreased on the 5th day. Captures in the control traps did not differ across days.

In the second field test, significantly more stable flies were captured from Broce traps baited with all three concentrations of 1-octen-3-ol at the first day, compared to the

control traps (Figure 6A; $F = 5.12$, $d.f. = 3, 16$, $P < 0.05$). On the second day, only the two highest doses (500 μg and 2500 μg) caught more flies than the control traps and traps baited with 100 μg of 1-octen-3-ol ($F = 3.76$, $d.f. = 3, 16$, $P < 0.05$). For phenol, the two highest doses caught more stable flies than the control traps in both days (Figure 6B; $F_{\text{day-1}} = 5.15$, $d.f. = 1, 32$, $P < 0.05$; $F_{\text{day-2}} = 3.79$, $d.f. = 1, 32$, $P < 0.05$). Comparing the effectiveness of attracting stable flies between 1-octen-3-ol and phenol, the number of stable flies caught in the 1-octen-3-ol baited traps exceeded those in traps baited with phenol, except those baited with 100 μg on the second day ($P < 0.0003$, Figure 6).

Field experiment 3 evaluated mixtures of phenol and *m*-cresol and *p*-cresol. Traps baited with combination of phenol (2500 μg) and *m*-cresol (100 μg), phenol (2500 μg) and *p*-cresol (100 μg), and phenol (2500 μg) alone captured significantly more stable flies compared to control (hexane) for day 2 (Figure 7, $F_{\text{day-2}} = 3.55$, $d.f. = 3, 16$, $P < 0.05$). On day 1, the mixture but not phenol alone captured more stable flies than the control. (Figure 7; $F_{\text{day-1}} = 4.48$, $d.f. = 3, 16$, $P < 0.05$;). There were no significant differences in the number of stable flies caught among phenol (2500 μg) and *m*-cresol (100 μg), phenol (2500 μg) and *p*-cresol (100 μg), and single phenol (2500 μg) on day 2. Comparing the number of stable flies captured between day 1 and day 2 revealed that significantly more stable flies were caught on day 2 than day 1 ($T_{(\text{phenol} + m\text{-cresol})} = 2.02$, $P < 0.05$; $T_{(\text{phenol} + p\text{-cresol})} = 2.42$, $P < 0.05$; $T_{(\text{phenol})} = 2.05$, $P < 0.05$).

DISCUSSION

Ruminants are preferred hosts for haematophagous insects due to their habit of herding and their sessile nature. Jeanbourquin and Guerin (2007) analyzed volatiles from the rumen digesta of cows and they found dimethyl trisulfide, butanoic acid, *p*-cresol,

skatole and especially 1-octen-3-ol that were attractive to stable flies. Manure odor has also been found to attract stable flies, especially gravid females, to oviposition sites (Broce and Haas 1999, Jeanbourquin and Guerin 2007). Among the volatile chemicals identified from manure are carboxylic acids which are host location attractants for mosquitoes (Knols et al. 1997, Bosch et al. 2000, Costantini et al. 2001). Broce and Haas (1991) found that gravid female stable flies prefer aged cow manure which produces twice the concentration of volatile carboxylic acids as those in fresh manure (Miller and Varel 2001). Our gas chromatography analysis of cow manure slush identified 8 compounds: acetic acid, propanoic acid, butanoic acid, phenol, *p*-cresol, *m*-cresol, 4-ethylphenol, and indole.

Tangtrakulwanich et al. (2011) found that acetic acid, phenol, *p*-cresol and indole evoked electroantennogram (EAG) responses from stable flies. Acetic acid and propanoic acids are known to be important in host location by a number of haematophagous insects (Jeanbourquin 2006). Among all chemical stimulating volatiles, 1-octen-3-ol has been documented as a strong attractant for various haematophagous insects (Hall et al. 1984, French and Kline 1989, Kline et al. 1990, Gibson and Torr 1999). In our behavioral olfactometer assay study, 1-octen-3-ol (100 µg) elicited greatest stable fly upwind movement of the single compounds tested compared to control (hexane). However, Alzogaray and Carlson (2000) found in a triple cage olfactometer that 1-octen-3-ol was not attractive to stable flies. Phenol is reported as another attractant to haematophagous insects such as stable flies (Mihok et al. 2007) and tsetse flies, *Glossina morsitans* and *G. pallidipes* in Zimbabwe (Vale et al. 1988). Results from our olfactometer study showed that phenol alone was not attractive to stable flies (Figure 4). *p*-Cresol and *m*-cresol elicit

significant EAG responses in stable flies (Hassanali et al. 1986, Bursell et al. 1988), but surprisingly they appear to be not attractive, or even as behavior antagonists for stable flies while testing single *p*-cresol and *m*-cresol by themselves in our olfactometer assay. But combinations of phenol (100 µg) with *p*-cresol (4 µg) or *m*-cresol (4 µg) were attractive to the stable flies in the olfactometer test (Figure 4).

The volatiles I tested are associated with cattle rumen digesta, cow manure slush and cattle urine. For example, phenol, *p*-cresol and *m*-cresol are components of urine volatiles. Phenol developed from tyrosine in the presence of microbial enzyme tyrosine ammonia lyase (Brot et al. 1965). *p*-Cresol and *m*-cresol were also detected in cow manure slush and is also considered to be associated with microbial degradation (Hopper and Taylor 1975). Mohammed et al. (2003) reported that *p*-cresol originates from tryptophan degradation by rumen bacteria. Bursell et al. (1988) found *p*-cresol was a major component of cattle urine (Birkett et al. 2004, Jeanbourquin 2006). 1-octen-3-ol, and emanation of terrestrial mammals (Hall et al. 1984, Raymer et al. 1985), is a secondary alcohol derived from 1-octene that acts as a kairomone to host seeking haematophagous insects, such as mosquitoes (Cork and Park 1996, Takken and Kline 1989) and tsetse flies (Hall et al. 1984).

Broce traps (Broce 1988) with alsynite panels are attractive to stable flies in part because they reflect UV light in a range of 360 to 420 nm as visual cues (Agee and Patterson 1983). CO₂ attracts stable flies to host location (Gatehouse and Lewis 1973, Gillies 1980, Vale 1980) and Cilek (1999) collected 25 times more flies when Alsynite[®] cylinder traps were modified to emit CO₂. He also had a six-fold increase in catches when the traps were baited with a 4:1:8 mixtures of octenol, propylphenol, and methyl phenol.

My field trapping results indicate that Broce traps with a lure of 1-octen-3-ol were more attractive to stable flies than the traps baited with control or phenol (Figure 6). This concurs with the findings of Holloway and Phelps (1991) and Mihok et al. (1995) who found that 1-octen-3-ol significantly increased stable fly catches in traps. However, in a study carried by Mihok et al. (1995), 1-octen-3-ol did not appear to increase catches of stable flies. Also, in field studies conducted by Mullen et al. (1995) and Cilek et al. (1999), 1-octen-3-ol failed to attract stable flies to cylinder traps. Mullen et al. (1995) speculated that the lack of responses in their study may have been due to the close proximity of the traps to large numbers of cattle. However, Cilek et al. (1999) argued that host proximity was not a factor in their study since the closest cattle were along distant (nearly 200 km distant) away. In my study, the 1-octen-3-ol baited traps located approximately 100 meters from the closest animal pen. The differences between my study and the studies where 1-octen-3-ol was not attractive may be related to the trap composition, physiological state of the flies, their geographic variation, bait concentration and carrier used for trapping test, as well as different environment parameters such as wind speed and wind direction.

1-octen-3-ol was a better attractant than phenol in field trials (Figure 6). Mihok and Mulye (2010) had similar results, reporting that 1-octen-3-ol was better in attracting horseflies than phenol. They studied responses of tabanids to Nzi traps baited with 1-octen-3-ol, cow urine and phenols in Canada and found that catches of horseflies, *Hybomitra lasiophthalma* (Macquart), *Tabanus similis* Macquart and *Tabanus quinquevittatus* Wiedemann (Diptera: Tabanidae) were increased by 1.5-2.6, 1.4-2.0 and 1.4-1.9 times, respectively in traps with 1-octen-3-ol compared to phenol or cow urine.

Mihok and Mulye (2010) also found that fly catches were not significantly different when traps were baited with phenol alone compare to control. Kline et al. (1990) reported that 1-octen-3-ol was an effective lure for the midge *Culicoides furens* (poe), whereas phenol was less effective. This may explain why phenol itself was not very attractive in the present study. From my olfactometer study and field trapping experiments, phenol is not attractive especially when tested at higher doses. There was a significant increase in collections of stable flies when 1-octen-3-ol was released at a 0.2-2.0 mg/hr. but only when 1-octen-3-ol was used by itself (Mihok et al. 1995). These results differ Cilek's (1999) findings that 1-octen-3-ol must be used in conjunction with phenols or urine. The mixture 1:1 of 1-octen-3-ol and phenol was tried for this study in the first experiment and the result showed that the mixture did not work well compared to single 1-octen-3-ol, or phenol itself (Figure 4). However, the ratio of the mixture used in this study was different than the ratio used in Cilek (1999). Despite the differences in number of flies captured in the lure baited traps, the longevity of both 1-octen-3-ol and phenol was found to be similar, about 3-5 days (Figure 5).

The addition of either *p*-cresol or *m*-cresol to phenol increased trap captures in the field than phenol alone but the combination of phenol and *m*-cresol captured the most stable flies ($P < 0.03$). The additive effect of combining more than a single compound to enhance fly captures has been noted in the past. Kyorku et al. (1990) studied testse *Glossina longkpennis* Corti in south-west Kenya and developed an effective trap/odor bait system. They found that neither acetone nor cow urine by themselves significantly increased trap catches. But combinations of acetone or cow urine with *p*-cresol, 3-n-propyl phenol and 1-octen-3-ol, were significantly more effective. Recently, Mihok and

Mulye (2010) demonstrated that traps baited with 4-methylphenol (*p*-cresol) or 3-n-propylphenol were ineffective in catching horseflies, *Hybomitra lasiophthalma*. But when traps were baited with cow urine (mixtures of more than one phenol) or cow urine and 1-octen-3-ol, catches of horseflies increased dramatically (1.5-2.6 times). Whether phenols are attractants or deterrents varies by species (Vale et al. 1988). 4-methylphenol (*p*-cresol) added to 3-n-propylphenol, for example, increased trap captures of *Glossina pallidipes* but reduced those of *Glossina morsitans* (Weldon and Carroll 2007). The mixture of phenol and *m*-cresol (25:1) provided the best trap catches of stable flies and was an effective attractant in similar trials for this study.

In conclusion, understanding the behavioral responses of stable flies to host associated volatiles is important as this knowledge could lead to improve monitoring and control technologies using attractants as part of a push pull strategy. Another interesting aspect of this present work is the synergism between *m*-cresol and phenol. Phenol alone was only slightly attractive but as a mixture with *m*-cresol, was very attractive to stable flies in both laboratory and field studies. This study demonstrated that mixtures of semiochemicals are more efficient stable fly attractants than single compounds. However, additional studies employing combinations of semiochemicals are needed to investigate the synergistic, additive or antagonistic effects of natural kairomones on stable fly movement. The better understanding of evaporation rates and degradation of attractant which affects trap efficiency and stable fly suppression is necessary.

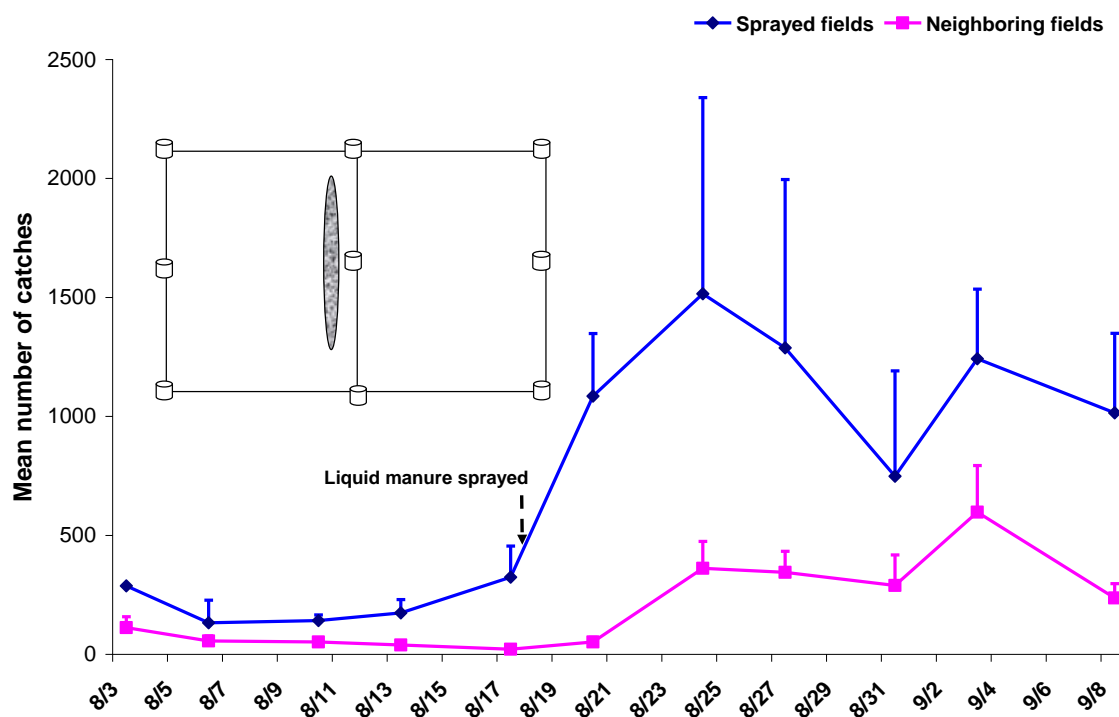
Figure 1

Figure 1 Comparisons of number of stable flies caught in traps between the manure/slush sprayed areas and those of their neighboring areas in UNL-ARDC, 2009.

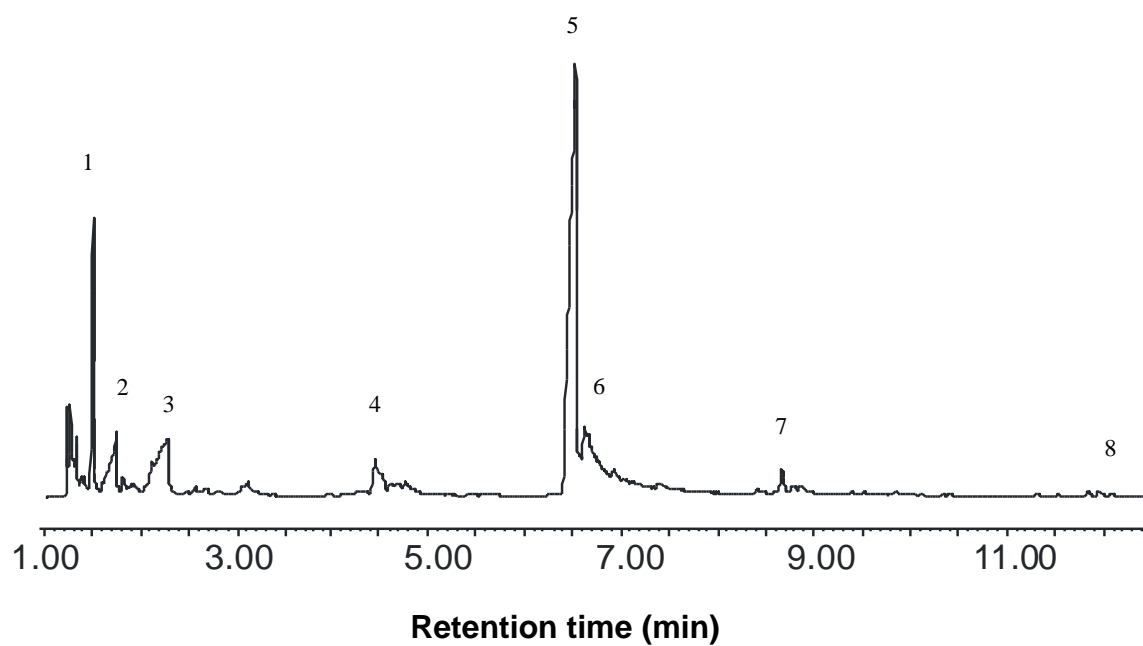
Figure 2

Figure 2 Gas chromatographic traces of fresh cow manure slush volatiles (1. acetic acid, 2. propanoic acid, 3. butanoic acid, 4. phenol, 5. *p*-cresol, 6. *m*-cresol, 7. 4-ethylphenol, 8. indole)

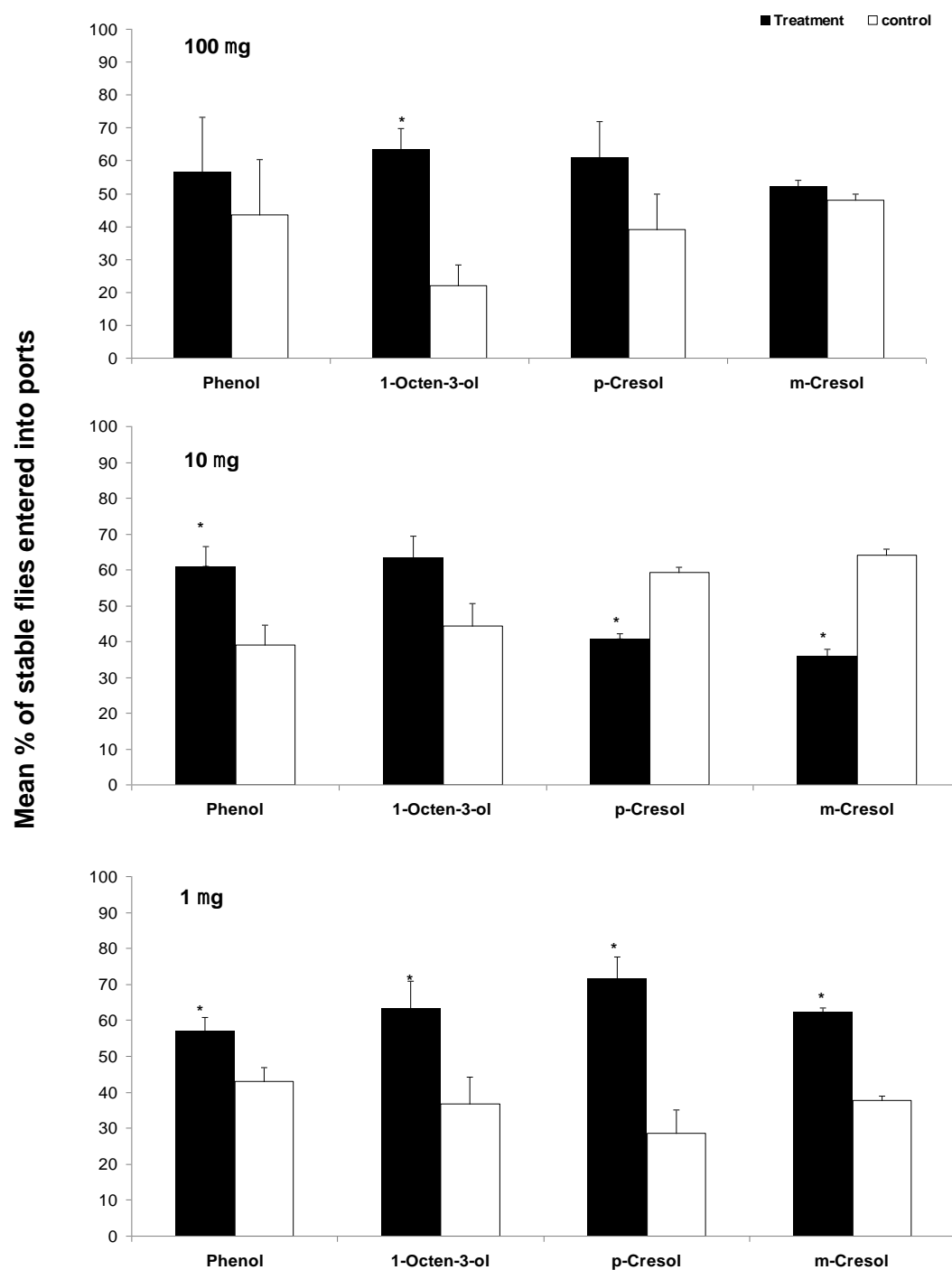
Figure 3

Figure 3 Mean % of stable flies observed into treatment or control ports of a single cage olfactometer within 5 minutes of exposure to host associated volatiles at three concentrations (\pm S.E., $N = 180-200$). Stars on top of bars indicate significant differences (Student's T -test. $P < 0.05$)

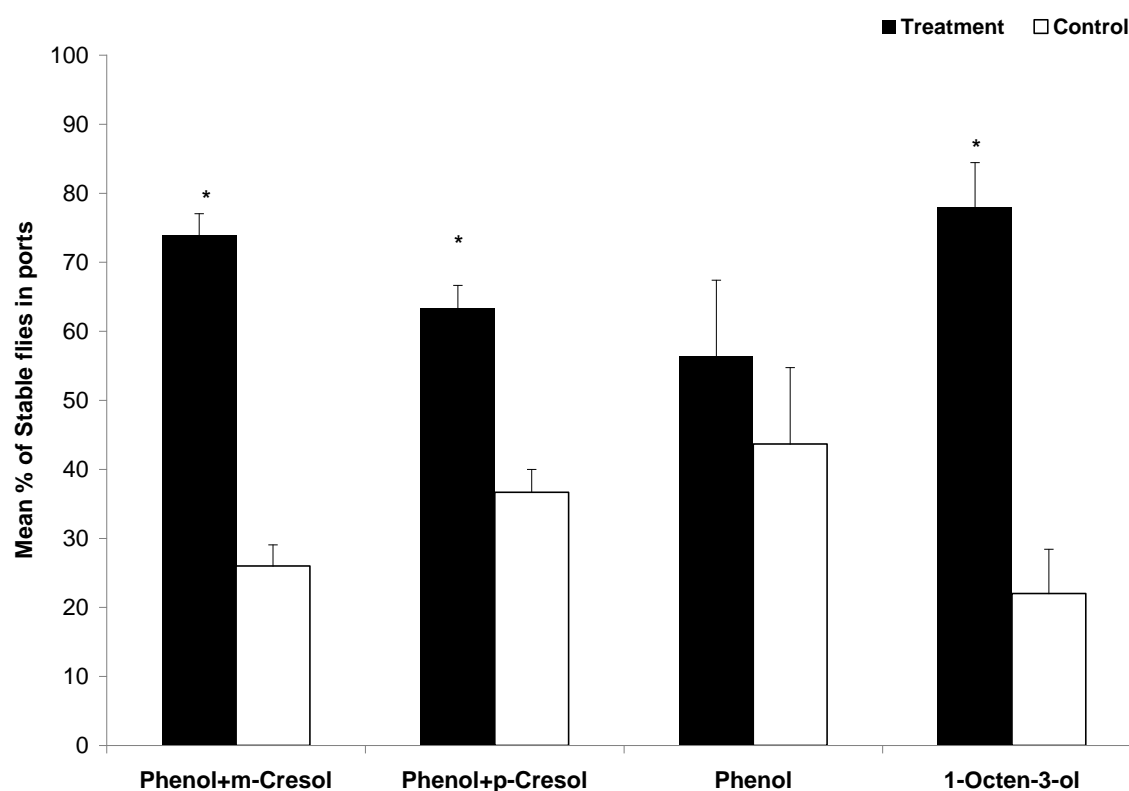
Figure 4

Figure 4 Mean % of stable flies observed in treatment and control ports of a single cage olfactometer within 5 minutes of exposure to host associated volatile compounds (phenol and 1-octen-3-ol (100 μ g) and *m*-cresol and *p*-cresol (4 μ g) (\pm S.E., $N= 180-200$ in each comparison) . Stars on top of the bars indicate significant differences (Student's *T*-test, $P < 0.05$).

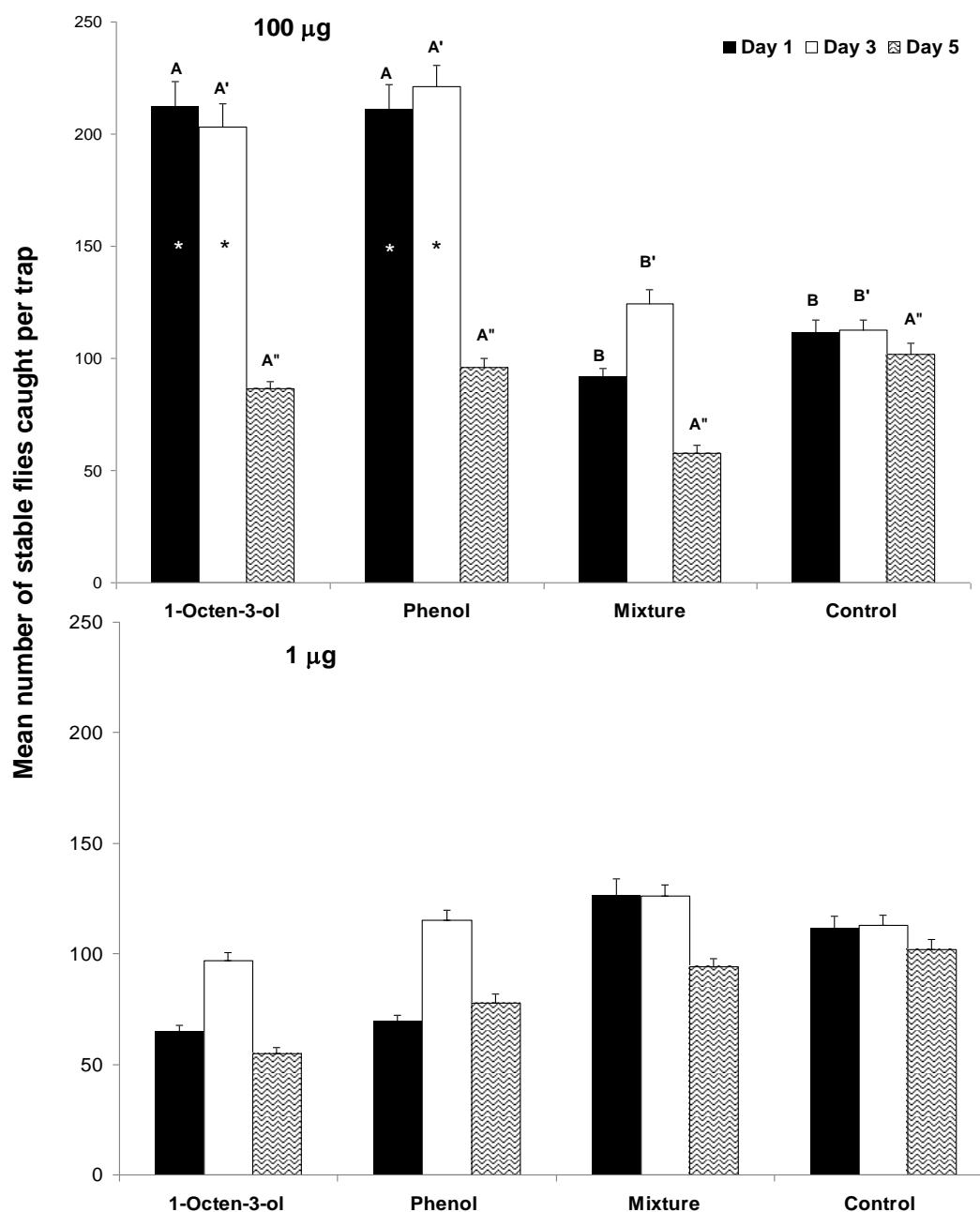
Figure 5

Figure 5 Mean number of stable flies caught over a three day period in Broce traps baited with 1-octen-3-ol or phenol or a mixture of both at low (1µg) and high (100 µg) concentrations. Means by day and concentration with different letters and symbols above the bars are significantly different ($P < 0.05$, ANOVA followed by least-square means, SAS version 9.1). * indicates significant difference in mean number of stable flies caught for each compound among days 1, 3, and 5 by concentration ($P < 0.05$, ANOVA followed by least-square means, SAS version 9.1).

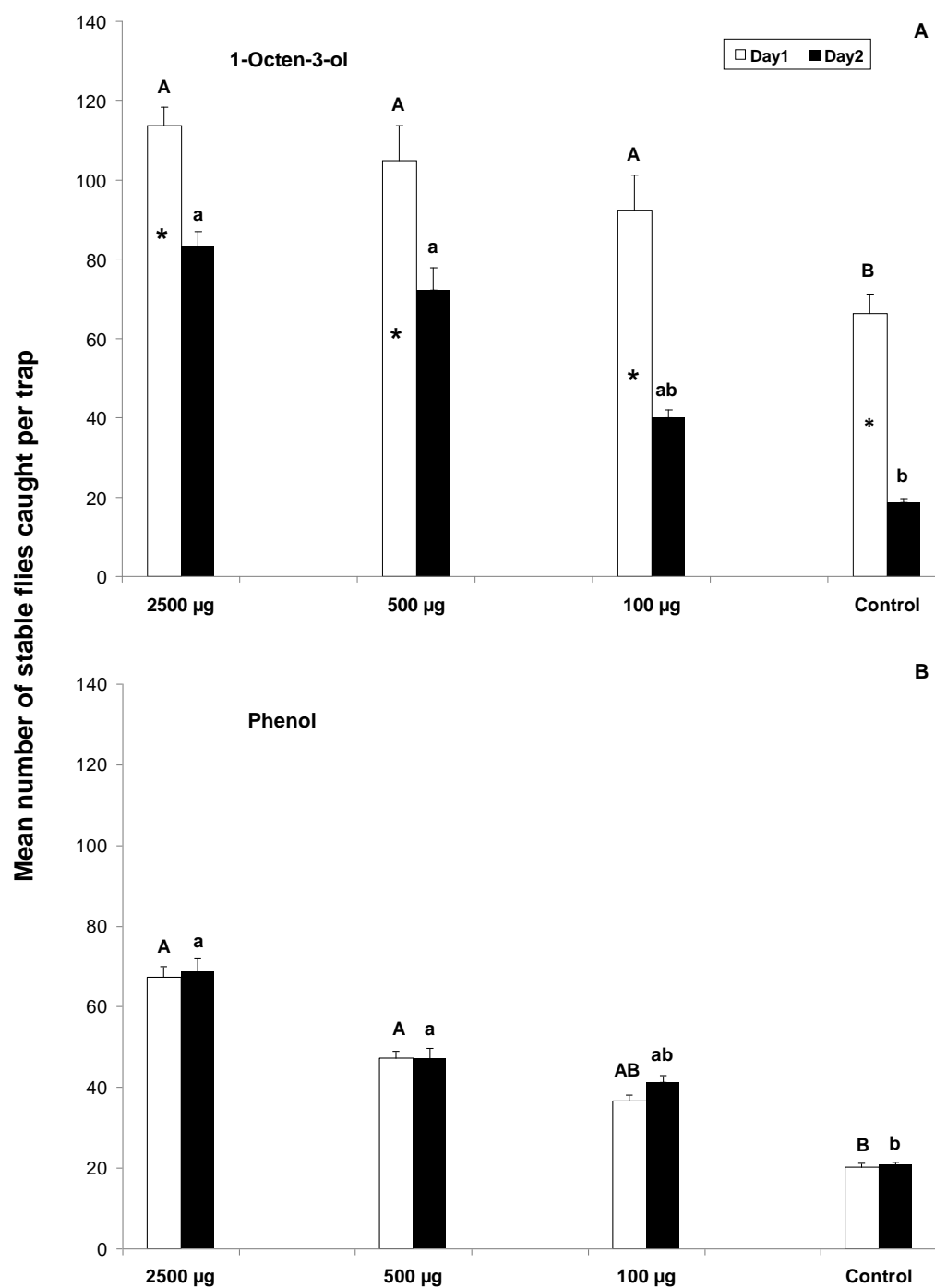
Figure 6

Figure 6 Mean number of stable flies caught in Broce traps baited with different concentrations of 1-octen-3-ol and phenol. Data were collected on day 1 and 2. Means within a day and for a test compound with different letters above the bars are significantly different ($P < 0.05$, ANOVA followed by least-square means, SAS version 9.1). * indicated significant differences in mean number of stable flies caught for each compound and each concentration between days 1 and Day 2 ($P < 0.05$, Student t test).

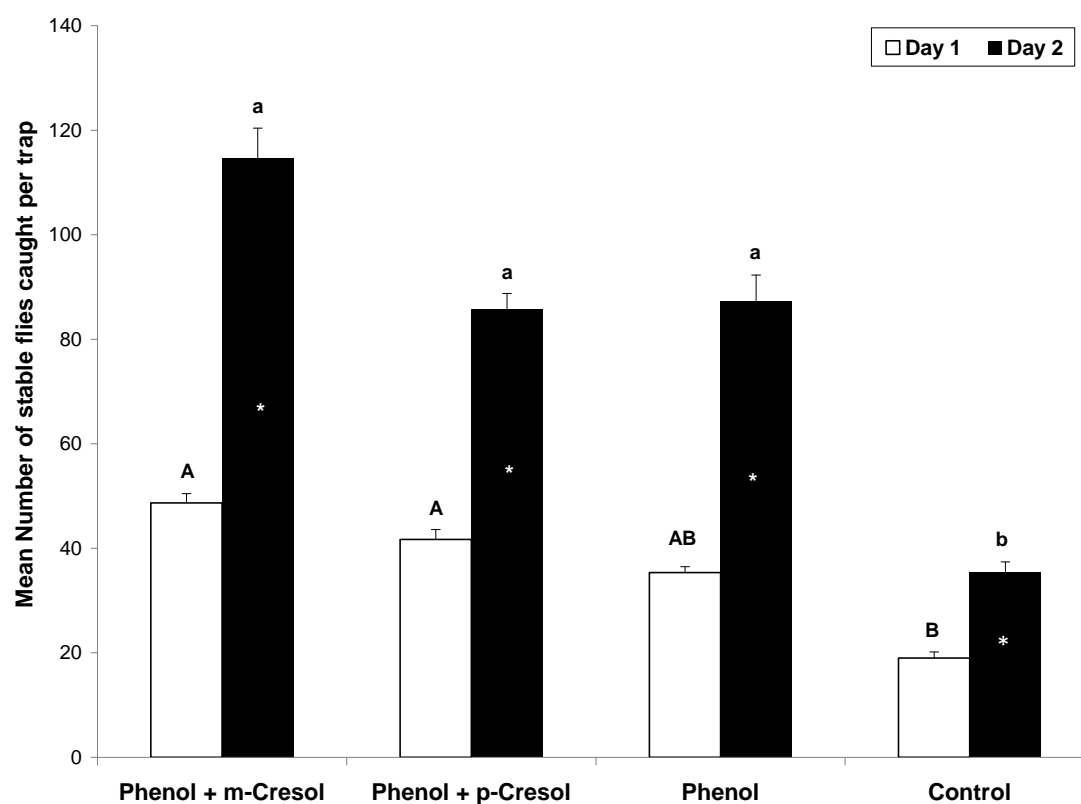
Figure 7

Figure 7 Number of stable flies caught in Broce traps baited with 2500 μg phenol alone or with 100 μg *m*-cresol or 100 μg *p*-cresol, or with hexane control. Data were collected on days, 1 and 2. Means by day with different letters above the bars are significantly different at $P < 0.05$ (ANOVA followed by least-square means, SAS version 9.1). * indicated significant differences in mean number of stable flies caught between day 1 and day 2.

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